CATALYSIS OF IRON CORE FORMATION IN ESCHERICHIA COLI BACTERIOFERRITIN

by

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ABSTRACT

Iron is an essential element for almost all life, so iron homeostasis is an important concern for most living organisms. The chemical properties of iron as represented by the low aqueous solubility of ferric iron and the toxicity of hydroxyl radicals it can produce by means of the Fenton reaction make achievement of iron homeostasis both challenging and crucially important. Bacterioferritin (BFR) is a bacterial member of the ferritin family of proteins that stores iron as a microcrystalline ferric hydroxide core of ~2700 iron atoms. This core is surrounded by 24 identical protein subunits, each possessing a dinuclear iron centre that catalyzes the oxidation of Fe$^{2+}$ to Fe$^{3+}$. This structure affords storage, solubility and bioavailability of iron. To improve our incomplete knowledge of the mechanism of iron core formation, the properties of an assembly variant (Glu128Arg/Glu135Arg) and the wild-type of Escherichia coli BFR have been characterized by X-ray crystallography, site-directed mutagenesis, and iron oxidation kinetics. The crystal structure of the variant included two ethylene glycol (EG) molecules adjacent to the dinuclear (ferroxidase) site that catalyzes iron oxidation. One EG resides in the ferroxidase pore that provides a route from the solvent to the ferroxidase site. The other EG resides at the inner surface of the protein where the iron core presumably binds and is surrounded by three acidic residues: Glu47, Asp50, and Asp126. Kinetics studies revealed that Glu47Gln, Asp50Asn and Asp126Asn substitutions in the assembly variant and the wild-type 24-mer retarded iron core formation and that Glu47 is important in iron oxidation at the ferroxidase site whereas Asp50 and Asp126 are important for iron core nucleation. The 3-fold channel, 4-fold channel, B-site channel and the ferroxidase pore of
BFR are possible routes of iron entry for core formation, but disruption of each of these sites individually in the 24-mer did not alter the kinetics of iron core formation. The intermediate states of the dinuclear site during iron oxidation are not well defined, but fast formation and decay of a $\mu$-1,2-peroxodiferric intermediate ($\lambda_{\text{max}} = 650$ nm) has been proposed. This intermediate was detected by multi-wavelength stopped flow kinetic analysis of wild-type BFR.
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<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>BFD</td>
<td>bacterioferritin associated ferredoxin</td>
</tr>
<tr>
<td>BFR</td>
<td>bacterioferritin</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
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<tr>
<td>Cr(TREN)</td>
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<td>DNA binding protein from starved cells</td>
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<td>extended X-ray absorption fine structure</td>
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<td>Fur</td>
<td>ferric uptake regulator</td>
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<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
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<tr>
<td>MES</td>
<td>2-(N-morpholino)ethanesulfonic acid</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
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<tr>
<td>NHE</td>
<td>normal hydrogen electrode</td>
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<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
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<tr>
<td>PAR</td>
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<td>Abbreviation</td>
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<tr>
<td>PBP</td>
<td>periplasmic binding protein</td>
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<td>SSRL</td>
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<td>TEV</td>
<td>tobacco etch virus</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>UV-Vis</td>
<td>ultraviolet-visible</td>
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CHAPTER I – INTRODUCTION

1.1 Iron in biology

Iron is an element which is vital for all life on Earth with rare exceptions (e.g., *Borrelia burgdorferi* and lactic acid bacteria which use manganese and cobalt instead of iron (Archibald 1983; Pandey *et al.* 1994; Weinberg 1997; Imbert and Blondeau 1998; Posey and Gherardini 2000)). Iron is incorporated in a vast number of proteins that function in essential biological processes such as photosynthesis, cellular respiration, oxygen transportation, DNA synthesis and nitrogen fixation (Crichton and Ward 1992).

In the Earth’s crust, iron is the second most abundant metal and fourth most abundant element. Although iron can exhibit oxidation states ranging from -II to +VI, iron (II) and iron (III) are the two most common biological forms (Crichton and Pierre 2001). The large diversity of functions exhibited by iron-containing proteins stems from the ability of proteins to tune the reduction potential of the iron(II/III) redox couple over a large range (-0.5V to 0.6V) by providing an appropriate iron binding environment (Andrews 1998; Crichton and Pierre 2001). Iron can be incorporated into proteins as iron-sulfur clusters, mononuclear iron sites, dinuclear iron sites, mixed metal dinuclear sites, and heme. The availability and usefulness of iron in biological processes have resulted in its assimilation into life processes at an early point in evolution (Beinert *et al.* 1997).

However, challenges of utilizing iron arose after the introduction of dioxygen into the atmosphere by photosynthesis.

The presence of atmospheric dioxygen created two major problems for the metabolic use of iron. Specifically, the oxidizing environment created by atmospheric
oxygen resulted in the shift from predominance of ferrous iron to predominance of ferric iron. This change was significant because in aqueous solution at neutral pH, iron(II) is soluble (0.1 M) and iron(III) is insoluble (1.4x10^{-9} M)(Chipperfield and Ratledge 2000; Andrews et al. 2003). This decrease in iron solubility decreased the bioavailability of iron so that it became a limiting nutrient. In addition, the combination of dioxygen and iron(II) in a reducing biological environment results in the oxidation of iron(II) to iron(III) and reduction of dioxygen to species such as superoxide, hydrogen peroxide and water. Hydrogen peroxide formed in this manner can react with iron(II) to produce hydroxyl radicals through the Fenton reaction (Kehrer 2000):

\[ \text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{OH}^- + \text{OH}^\cdot \]

Hydroxyl radicals (OH\(^\cdot\)) are highly reactive and can readily oxidize biological macromolecules such as nucleic acids, proteins and lipids. Therefore, protective or detoxifying mechanisms must come in play to prevent iron and oxygen from reacting freely. Ultimately, living organisms are faced with the challenge of acquiring iron in iron scarce environments and with storing iron safely in a non-reactive form when it is available.

### 1.2 Bacterial strategies for iron acquisition

Microorganisms have developed strategies to deal with the limited bioavailability of iron. For example, soluble ferrous iron in the environment is directly transported into the cells via the Feo system (Hantke 1987; Kammler et al. 1993; Cartron et al. 2006).
The Feo system is typically associated with bacteria growing in anaerobic-microaerophilic or low pH environments which would favour the ferrous form of iron over the ferric form. Ferrous iron uptake is increased by ~4-fold in a fur mutant, which suggests that the Feo system is repressed by Fur (Hantke 1987). The Feo system in *E. coli* is composed of FeoA, FeoB, and FeoC (Kammler *et al.* 1993; Hantke 2003; Cartron *et al.* 2006). FeoA is an 8.4 kDa hydrophilic SH3-domain protein most likely found in the cytosol, FeoB is an 84.5 kDa protein with a cytosolic N-terminal G-protein region and a transmembrane C-terminal region with two Gate motifs, and FeoC is an 8.7 kDa protein with a winged-helix region which might contain an iron-sulfur cluster (Loewen *et al.* 1999; Gajiwala and Burley 2000; Marlovits *et al.* 2002; Cartron *et al.* 2006). Ferrous iron is believed to diffuse through the bacterial outer membrane through porins before being actively transported through the inner membrane by FeoB. The roles of FeoA and FeoC are unclear and are not found in some bacteria such as *Helicobacter pylori* (Velayudhan *et al.* 2000; Hantke 2003).

The bacterial acquisition of ferric iron has been more thoroughly studied (Figure 1.1). Ferric iron is solubilised by the secretion of siderophores, small iron chelators that specifically bind iron with high affinity. Over 500 different siderophores have been identified (Boukhalfa and Crumbliss 2002). A commonality observed amongst all siderophores is the use of the following chelating groups: α-hydroxycarboxylic acid, catechol, and/or hydroxamic acid (Winkelmann 1990; Winkelmann 2002). The iron-siderophore complexes are recognized and transported through the outer membrane by receptors that consist of a 22 anti-parallel β-stranded β-barrel and an N-terminal cork domain (Ferguson *et al.* 1998; Ferguson *et al.* 2000; Ferguson *et al.* 2001; Ferguson *et al.*
The TonB-ExbB-ExbD complex, which is located at the inner membrane, couples the inner membrane proton motive force with the active transport of iron-siderophores through the outer membrane receptors (Kadner 1990; Postle 1993; Larsen et al. 1996; Stojiljkovic and Srinivasan 1997). Periplasmic binding proteins (PBP) bind and shuttle the iron-siderophore complexes to the inner membrane. ATP-binding cassette (ABC) transporters couple the energy from ATP hydrolysis with the transport of iron-siderophore complexes through the inner membrane (Koster 2001). Once inside the cytoplasm, iron is released from the siderophores either by iron reduction or siderophore breakdown.

The competition for iron between host and bacteria is an important factor in the success of pathogenic organisms. Host iron-binding proteins such as transferrin and lactoferrin have been proposed to have anti-bacterial activity as a result of their ability to sequester iron from bacteria (Payne 1993; Weinberg 1993; Andrews et al. 2003). Nevertheless, siderophores secreted by bacteria can effectively compete with host iron-binding proteins for iron. Receptors on some bacterial cells recognize these iron-binding proteins and can use them as an iron source after iron extraction (Gray-Owen and Schryvers 1996). Upon release of host heme and hemoglobin to the blood, they are bound by hemopexin and haptoglobin, respectively, to prevent the use of heme by bacteria as an iron source. However, receptors on some bacteria can recognize heme, myoglobin, hemoglobin, hemoglobin-haptoglobin, heme-albumin, and heme-hemopexin complexes for extraction of the heme before transport into the cell.
Figure 1.1. Overview of bacterial ferric iron uptake. (A) Various iron uptake pathways in gram-negative bacteria that involve an outer membrane receptor, PBP, an ABC transporter, and the TonB system. (B) Iron uptake in gram-positive bacteria involves a membrane-anchored binding protein that resembles PBP and an ABC transporter. Reproduced from (Krewulak and Vogel 2008) with permission from Elsevier.
Bacteria release hemolysins, cytolysins, and proteases to increase access to heme and heme-containing proteins (Stoebner and Payne 1988; Daskaleros et al. 1991; Pickett et al. 1992; Lagergard et al. 1993; Simpson and Oliver 1993). Bacteria also release hemophores that facilitate the delivery of heme from heme complexes to the bacterial cells. In the cytoplasm, heme may be degraded by heme oxygenase-like proteins to extract the iron (Schmitt 1997; Wilks and Schmitt 1998; Zhu et al. 2000; Zhu et al. 2000).

Similar to transport of iron-siderophore complexes, the transport of heme and ferric iron involve outer membrane receptors, the TonB complex, PBPs, and ABC transporters (Figure 1.1).

1.3 Overview of ferritin

The almost universal need of living organisms for iron is reflected in the nearly ubiquitous nature of ferritin and ferritin-like proteins which are found in archaea, bacteria and eukaryotes. Ferritin is an iron storage protein which traps iron inside its shell to store it in a compact form that also limits the amount of “free” iron that would otherwise engage in Fenton chemistry and promote formation of reactive oxygen species. Ferritin stores and maintains the normally insoluble iron(III) within a soluble protein shell that envelopes and stores iron for use in times of low iron availability. Mammalian ferritins are comprised of twenty four subunits that assemble to form a hollow, spherical shell of varying ratios of two types of subunits, H-chain (heavy) and L-chain (light). With an inner and outer diameter of approximately 80 Å and 120 Å respectively, mammalian ferritin has a predicted iron storage capacity of up to 4500 iron atoms per molecule (Ford et al. 1984). Each subunit is ~18-21 kDa in mass and folds to form four main antiparallel
alpha helices (four-helix bundle) and one small alpha helix at the C-terminal (Lawson et al. 1991).

The H-chain subunit is characterized by the presence of a dinuclear iron site located at the centre of the structure. This site binds iron(II) and catalyzes its oxidation by dioxygen to form iron(III). The L-chain subunit lacks the dinuclear iron site but has a larger number of acidic side chains on the surface facing the cavity of the protein shell, that provide nucleation sites for iron core formation. The ratio of H-chain and L-chain subunits in ferritin found in the human tissues varies from tissue to tissue and the function of the protein. Ferritin with a high L-chain content is found in organs that store iron, such as the liver and spleen, and typically have a higher level of iron (>1500 Fe/molecule) than ferritin with higher levels of H-chain ferritin. Ferritin with high H-chain content is found in organs such as the heart and brain that store relatively low amounts of iron (<1000 Fe/molecule).

1.4 Ferritin and ferritin-like proteins in microorganisms

*Escherichia coli* (*E. coli*) contains several ferritin or ferritin-like proteins: bacterial ferritin (FtnA and FtnB), bacterioferritin (BFR) and DNA binding protein from starved cells (Dps). All these proteins are generally composed of one type of subunit as opposed to two as in mammalian ferritin. Nevertheless, two types of subunits are found in *Pseudomonas aeruginosa* BFR and two *bfr* genes possibly encoding two types of subunits are found in *Magnetospirillum magnetotacticum*, *Neisseria gonorrhea*, and *Synechocystis* (Moore et al. 1994; Bertani et al. 1997). *E. coli* FtnA is composed of subunits (~19.5 kDa) which are most like the H-chain subunit of mammalian ferritin due
to the presence of a dinuclear iron site. The amino acid ligands in the dinuclear iron site of mammalian ferritin are conserved in the dinuclear site of FtnA, but interestingly, a third iron binding site exists in FtnA which is believed to cause some of the mechanistic differences observed between these two proteins. FtnB lacks some of the crucial residues of the dinuclear iron site, is less well characterized than FtnA, and is not believed to be capable of forming heteropolymers with FtnA because it does not have the inter-subunit bonding residues seen in FtnA.

Dps is unique in that the spherical shell is an assembly of just 12 subunits in comparison to 24 subunits as in the other ferritins (Grant et al. 1998). Although Dps is capable of binding and storing iron, its function is thought to be a response to oxidative stress. Dps also binds DNA non-specifically. Although this protein possesses dinuclear iron sites, these sites are not located in the centre of each subunit but are constructed between pairs of adjacent subunits with each subunit contributing residues that provide ligands for binding iron. Recently, the crystal structure of a Dps-like protein from the archaea Sulfolobus solfataricus has been determined (Gauss et al. 2006). Like Dps, the Dps-like protein forms dodecameric spherical structures, is expressed in response to oxidative stress, preferentially uses H₂O₂ for the oxidation of iron, and is believed to bind DNA. Interestingly, the Dps-like protein contains a dinuclear iron site at the centre of each subunit as in BFR and not at the interface of two adjacent subunits as in Dps.

BFR is also composed of subunits more similar to H-chain subunits because it contains dinuclear iron sites. The residues that form the dinuclear iron sites in BFR are slightly different from those that form the corresponding mammalian sites but are nevertheless active in binding and oxidizing iron(II). The effective iron-binding capacity
of BFR is \( \sim2700 \) iron atoms per BFR 24-mer (Baaghil et al. 2003). Interestingly, BFR is also characterized by 12 heme binding sites that are located between pairs of subunits. Each subunit contributes a surface methionyl residue for binding the heme iron in a symmetrical fashion to result in bis-methionine axial coordination (Cheesman et al. 1990). BFR from \textit{E. coli} is the focus of this dissertation.

1.5 Discovery of bacterioferritin

Cytochrome \( b_1 \), amongst other cytochromes, was studied by Keilin (1934) and Keilin and Harpley (1941). It was later purified by Deeb and Hager (1962; 1964) and Fujita \textit{et al.} (1963) from \textit{E. coli}. Subsequently, a cytochrome \( b \)-type protein that contained a large amount of iron was isolated from \textit{Azotobacter vinelandii} (\textit{A. vinelandii}) (Bulen \textit{et al.} 1973). Stiefel and Watt (1979) used electron microscopy to establish that cytochrome \( b_{557.5} \) from this organism has a structure similar to that of ferritins and consequently named it bacterioferritin. Similarly, Bauminger \textit{et al.} (1980) and Yariv \textit{et al.} (1981) isolated a protein from \textit{E. coli} that they referred to as bacterioferritin as the result of similarity of its structure to that of ferritin and Yariv (1983) later proposed that cytochrome \( b_1 \) and bacterioferritin are, in fact, the same protein. In time, cytochrome \( b_1 \) and bacterioferritin were purified, crystallized, and their X-ray crystallographic data were compared to establish that they are identical (Smith \textit{et al.} 1988). Hence, cytochrome \( b_1 \), cytochrome \( b_{557.5} \) and bacterioferritin are the same protein.
1.6 Structural features of bacterioferritin

1.6.1 Dinuclear iron site

Each BFR subunit contains one dinuclear iron site that is also regarded as the catalytic centre, that is responsible for catalyzing the oxidation of Fe(II) by dioxygen. This catalytic activity is generally referred to as ferroxidase activity. The dinuclear iron site of *E. coli* BFR is composed of one glutamyl, one histidyl and two bridging glutamyl residues that coordinate each iron atom (Figure 1.2C). Although the ligands appear to be equivalent for both iron atoms, the binding sites have non-symmetrical environments and consequently exhibit inequivalent binding affinities for metal ions. The iron atoms at the ferroxidase site are assumed to have a reduction potential above ~0 V (vs. NHE) because they are reduced by ascorbate (Le Brun *et al.* 1993b). The ferroxidase site is capable of binding metals other than iron including zinc, cobalt, manganese and uranium. Zinc is a known inhibitor of iron oxidation activity and is thought to do so by binding to the dinuclear iron site (Le Brun *et al.* 1995; Yang *et al.* 2000; Baaghil *et al.* 2003). Many crystal structures of BFR from different bacterial species have been obtained with metal ions other than iron bound at the dinuclear iron site. His130, one of the dinuclear site ligands, has been observed to exhibit alternate conformations that may be relevant to the mechanism of iron oxidation (Liu *et al.* 2004; Swartz *et al.* 2006).
The dinuclear iron site is a common motif found in a vast number of proteins with diverse functions. These diiron proteins are divided into at least four classes by Nordlund and Eklund (1995) based on their structural features. Class I diiron proteins, which include ribonucleotide reductase, methane monooxygenase, and stearoyl-acyl carrier...
protein $\Delta^9$-desaturase, are large helix-bundle proteins. Class II diiron proteins, which include ferritin, BFR, and rubrerythrin, are “simple” helix-bundle proteins. Both class I and II diiron proteins are involved in oxidation-hydroxylation reactions and contain a left-handed overhead connection found between two helix pairs. Class III diiron proteins are simple helix-bundle proteins without an overhead connection between the helices. This class includes hemerythrin and myohemerythrin which are involved in oxygen transport. Class IV diiron proteins, unlike the other classes, are $\alpha/\beta$ sandwiches and include purple acid phosphatases. Although these proteins have distinct functions, a common theme is the involvement of oxygen binding and/or activation (Edmondson and Huynh 1996).

BFR and other diiron proteins in class I, II, and III contain a four-helix bundle protein fold. In addition to the diiron proteins, the four-helix bundle fold is also found in other proteins either as an individual fold or as part of a larger structure (Harris et al. 1994; Kamtekar and Hecht 1995). Human growth hormone, cytochrome b562, granulocyte-macrophage colony-stimulating factor, repressor of primer, and T4 lysozyme are examples of proteins containing the four-helix bundle fold which are involved in various functions.

1.6.2 Heme binding site

BFR was thought to be a cytochrome when it was first isolated because it possesses heme. Twelve heme binding sites occur in bacterioferritin, and these sites give the protein a reddish color when heme is bound. For BFR from most species, iron-protoporphyrin IX is the form of heme that is bound, but iron-coproporphyrin III is found
in BFR from *Desulfovibrio desulfuricans*, an anaerobic bacterium (Romao et al. 2000). Each heme group is sandwiched between two subunits and exhibits bis-methionine axial ligation (Cheesman et al. 1990; Frolow et al. 1994). The methionyl residues provided by two separate subunits give a unique absorbance maximum at ~739 nm (Andrews et al. 1993; Andrews et al. 1995). This mode of heme binding was found exclusively in BFRs until the discovery of bis-methionine ligation in the surface protein Shp of *Streptococcus pyogenes* (Ran et al. 2007). Heme bound to BFR is oriented so that the heme propionate groups are exposed on the inner surface of the protein that faces the insoluble iron core.

The presence of heme in BFR is an intriguing matter as heme is normally present in proteins as an essential cofactor. No clear functional role has been established for heme in BFR, but there is evidence for its involvement in iron release, possibly through mediating the reduction of the iron core (Andrews et al. 1995). Heme-free *E. coli* BFR, obtained by replacing the methionyl residues that normally bind heme with alanine, accumulate four times as much iron *in vivo* as does the heme bound wild-type protein. Heme is not required for protein assembly or iron oxidation activity, both of which are the same *in vitro* for the heme-free variants and the wild-type protein. The reduction potential of heme in *A. vinelandii* BFR is -225 mV (vs. NHE) (Watt et al. 1986). This shifts to -475 mV when an iron core is present. The iron core has a reduction potential of -420 mV which raises possibilities of the interactions between the iron core and heme. The reduction potential for the iron-coproporphyrin III bound to *D. desulfuricans* is +140 mV which is relatively high compared to *A. vinelandii* (Romao et al. 2000). The reduction potential of heme bound to *E. coli* BFR has not been reported.
1.6.3 Iron entry channels

There are eight 3-fold symmetry channels, six 4-fold symmetry channels and twenty four B-site channels formed between subunits that are possible entry and exit routes for components of the iron core (Figure 1.3). The 3-fold channel in *E. coli* BFR is lined with the charged residues Asp109, Arg117 and Asp118. The 4-fold channel is lined with the polar residues Asn148 and Gln151. The B-site channel is lined with Asp132, Glu135, Thr136, and Asp139 with Asn34 and Glu66 from different subunits. The ferroxidase pore, located adjacent to the ferroxidase site, has also been proposed as a possible iron entry route. The ferroxidase pore opening is lined with Asn17, Val20, Ala21, Leu93, Gly97, with Asp96 close by. For comparison, the human H-chain ferritin 3-fold channel is lined with charged residues Glu134 and Asp131. The 4-fold channel is lined with mainly hydrophobic residues Leu165, Leu169 and His173. The ferroxidase pore is lined with Leu26, Tyr29, Ala30, Leu106 and Val110. The B-site channel is not observed in structures of mammalian ferritins (Carrondo 2003).

In human ferritin, the 3-fold symmetry channel is believed to play a significant role in the transport of iron into and out of the iron core. Substitution of residues lining the 3-fold symmetry channel lead to decreased ferroxidase activity (Treffry *et al.* 1993; Levi *et al.* 1996) and reduced iron binding at the ferroxidase site as determined by isothermal titration calorimetry and fluorescence quenching experiments (Bou-Abdallah *et al.* 2002; Bou-Abdallah *et al.* 2008). Inhibition of iron incorporation by terbium(III) and zinc(II) was shown to be achieved by binding and blocking the 3-fold symmetry channels. The inhibitory effect of Cr(TREN) has also been attributed to blockage of the 3-fold symmetry channel (Barnes *et al.* 2002; Barnes *et al.* 2003).
Figure 1.3. Inter-sub unit channels and ferroxidase pore in BFR. The subunits forming the 3-fold, 4-fold, and B-site channels and the ferroxidase pore are highlighted in orange, and the residues are coloured in red. Close-ups of the sites are shown on the right of the overall structure. (A) The 3-fold channel is lined with charged residues. (B) The 4-fold channel is lined with polar residues. (C) The B-site channel is lined with mainly acidic residues. (D) The ferroxidase pore is lined with mainly hydrophobic residues. The green spheres represent manganese ions in the dinuclear iron site. Figures generated from PDB ID: 1BFR.
Disruption of the 3-fold channel by mutagenesis, with chaotropic agents, peptides, or increased temperature increases the rate of iron release (Takagi et al. 1998; Jin et al. 2001; Liu et al. 2003; Liu et al. 2007). Based on electrostatic potential calculations, the 4-fold symmetry channel is postulated to form a proton wire that is important for maintaining electroneutrality (Takahashi and Kuyucak 2003). It is not known if BFR similarly uses the 3-fold channel for iron uptake. However, the Asp118Ala variant of the 3-fold channel did not exhibit a significant decrease in iron oxidation activity in BFR (Le Brun et al. 1995). Crystal structures of BFR from *A. vinelandii* showed the binding of barium and iron in the 4-fold symmetry channel, supporting involvement of this channel as an iron entry route (Liu et al. 2004; Swartz et al. 2006). Magnesium was also found in the B-site channels of *A. vinelandii* and *Mycobacterium smegmatis* (Swartz et al. 2006; Janowski et al. 2008). Electrostatic potential calculations support a role of the 3-fold channel in human H-chain ferritin as an entry route for iron (Douglas and Ripoll 1998; Takahashi and Kuyucak 2003). Calculation of the electrostatic potential surfaces of BFRs from *D. desulfuricans* and *E. coli* identified the B-site channels as possible routes of iron entry not found in mammalian ferritins (Figure 1.4) (Carrondo 2003; Macedo et al. 2003).
Figure 1.4. Electrostatic potential surfaces of ferritin and BFR. The electrostatic potential surface diagrams of human ferritin (A, B) and *E. coli* BFR (C, D) are shown. The protein is oriented so that the 4-fold channel (A, C) or 3-fold channel (B, D) is in the centre. Four and three B-site channels are visible in C and D, respectively, and one B-site channel is labelled in each diagram. Negative and positive surface potentials are coloured red and blue, respectively. Figures generated from PDB ID: 2CEI and 1BFR.
1.7 Bacterioferritin function

1.7.1 Physiological role of iron binding proteins in bacteria

BFR has been shown to play different roles in different species of bacteria, but in general, this protein is believed to be involved in iron storage and protection against oxidative stress. The exact physiological roles of this protein are difficult to define unambiguously owing to the presence of multiple types of iron binding proteins in bacteria. Gene knockout studies show FtnA to be important for iron storage in *E. coli* and *Porphyromonas gingivalis* (Abdul-Tehrani *et al.* 1999; Ratnayake *et al.* 2000). FtnA in *Erwinia chrysanthemi* 3937 and *Campylobacter jejuni* are important for both iron storage and protection against oxidation stress (Wai *et al.* 1996; Boughammoura *et al.* 2008). The roles of BFR in *E. coli*, *Erwinia chrysanthemi* 3937, and *Brucella melitensis* are not clear, but BFR in *Neisseria gonorrhoeae* and *Salmonella enterica* serovar Typhimurium are shown to play important roles in iron storage and protection against iron-mediated oxidative stress (Denoel *et al.* 1995; Abdul-Tehrani *et al.* 1999; Chen and Morse 1999; Velayudhan *et al.* 2007; Boughammoura *et al.* 2008). *E. coli* BFR reduces the production of hydroxyl radicals from the oxidation of ferrous ions by hydrogen peroxide *in vitro* (Bou-Abdallah *et al.* 2002). BFR catalyses the two electron reduction of hydrogen peroxide to water, thereby circumventing Fenton chemistry to prevent oxidative damage. BFR A from *Pseudomonas aeruginosa* is suggested to provide an iron source for the heme prosthetic group of KatA, a catalase that is important for protecting the cell against oxidative damage from hydrogen peroxide (Ma *et al.* 1999). Although Dps is also capable of binding iron, its main role is thought to be in protecting cells from oxidative damage. Two mechanisms have been proposed to account for this protective effect. Free
iron is preferentially oxidized by hydrogen peroxide over oxygen, thereby removing the reactants for the Fenton reaction. Dps also binds DNA to protect it physically from oxidative attack.

If BFR functions as an iron storage protein, the interesting question of how iron is released from the insoluble core becomes important. Bacterioferritin-associated ferredoxin (BFD) is a small, positively charged protein containing a [2Fe-2S] cluster, the gene for which (bfd) is typically associated with the bfr gene in bacterial chromosomes, and the corresponding proteins were shown to interact (Quail et al. 1996). The low reduction potential of -254 mV (vs. NHE) for E. coli BFD and its interaction with BFR suggest that BFD could be a reductant of BFR iron that could facilitate iron release. The reduction potentials of the heme and iron core are known for A. vinelandii BFR but not for E. coli BFR. It is also possible that upon binding of BFD with BFR, the reduction potentials change, as seen for the R2 subunit of ribonucleotide reductase and the hydroxylase domain of methane monooxygenase when they are bound by interacting proteins. Therefore, it is difficult to determine the flow of electrons without further studies. BFD expression is induced by iron starvation, which is the opposite of BFR. This relationship supports a model in which under conditions of limited iron availability, BFD is expressed to promote the release of iron from BFR. In D. desulfuricans, rubredoxin-2 (RD2) may be the source of electrons required for the release of iron from BFR. The bfr gene of D. desulfuricans is associated with the rd2 gene, and the corresponding proteins are also shown to interact in vitro. RD2 possesses an [Fe-(SCys)₄] centre with a reduction potential of +25 mV (LeGall et al. 1998). The RD2 protein has been shown to reduce the
iron-coproporphyrin III cofactor of *D. desulfricans* BFR which has a reduction potential of +140 mV as noted previously (Romao *et al.* 2000; da Costa *et al.* 2001).

### 1.7.2 Mechanism of iron loading in bacterioferritin

The binding and oxidation of iron by BFR occurs in at least three kinetically distinct phases (Figure 1.5) (Le Brun *et al.* 1993b). The first and fastest phase exhibits a half-life of ~50 ms (2.25 μM BFR, 112 μM Fe(II), pH 6.5, 30 ºC) and involves the reversible binding of Fe(II) at the dinuclear iron site. This kinetic phase was first detected by observation of spectroscopic changes in the 380-450 nm region following aerobic stopped-flow mixing of ferrous ammonium sulfate with iron-free BFR (Le Brun *et al.* 1993b). This binding of Fe(II) at the ferroxidase site apparently perturbs the heme environment sufficiently to induce a blue shift of the heme Soret band that is < 1 nm. The heme is unlikely to be reduced in this process because reduction of heme results in an 8 nm red shift of the Soret band that is accompanied by a substantial increase in molar absorptivity.

![Figure 1.5. The three kinetic phases of iron binding to bacterioferritin. Phase 1: Fe$^{2+}$ binds to apo-BFR; Phase 2: Fe$^{2+}$ bound to the dinuclear site is oxidized to Fe$^{3+}$; Phase 3: iron core formation proceeds as additional Fe$^{2+}$ is oxidized. The change in absorbance at 340 nm that is observed during aerobic addition of Fe$^{2+}$ to apo-BFR results from Phases 2 and 3 but is influenced by the kinetics of Phase 1.](image-url)
The Phase 1 reaction has also been studied by pH-stat experiments. In this work, the amount of base required to keep the pH constant as iron is added to the protein established that approximately two protons are released for every iron(II) that binds to the ferroxidase site (Yang et al. 2000). Similar observations had been made previously for the binding of zinc(II), manganese(II), and cobalt(II) to BFR (Le Brun et al. 1996). The Phase 1 reaction can be represented by the following equation where $P^Z$ represents the protein and $[\text{Fe(II)}_2-P]^Z$ represents the iron bound protein.

$$2 \text{Fe(II)} + P^Z \rightarrow [\text{Fe(II)}_2-P]^Z + 4 \text{H}^+$$

The second and slower phase ($t_{1/2} \sim 5 \text{ sec}$) involves the oxidation of the Fe(II) bound at the dinuclear iron site to Fe(III) by either dioxygen or hydrogen peroxide. The oxidation of iron is observed as an increase in absorbance at 340 nm that results from a broad ligand to metal (O $\rightarrow$ Fe(III)) charge transfer band. This spectroscopic change occurs over a period of $<20 \text{ sec}$ and can be observed by stopped-flow spectroscopy. The amplitude of the absorbance change varies linearly with iron concentration up to the point where the iron concentration approximately equals the amount of iron required to completely fill the ferroxidase site. At this point, the amplitude of the absorbance change reaches a plateau and does not increase further as the concentration of iron is increased.

The Phase 2 reaction has also been studied using an oxygen electrode (Bou-Abdallah et al. 2002). In these experiments, the kinetics of the reaction are followed by monitoring consumption of the dioxygen as the ferroxidase reaction proceeds. The stoichiometry of the dioxygen consumed in the reaction leads to the following equation.
where $[\text{Fe(III)}_2\text{O-P}]^Z$ represents some unknown form of the oxidized iron at the dinuclear iron site.

$$[\text{Fe(II)}_2\text{-P}]^Z + \frac{1}{2} \text{O}_2 \rightarrow [\text{Fe(III)}_2\text{O-P}]^Z$$

The third and slowest phase of this reaction ($t_{1/2}$ ~300 sec) involves the binding and oxidation of Fe(II) to Fe(III) at the inner iron core (Le Brun et al. 1993b). Phase 3 is also followed by monitoring the absorbance increase at 340 nm over a period of approximately 25 minutes, so this phase of core formation can be monitored spectrophotometrically without rapid mixing. Phase 3 occurs after Phase 2 and can be observed when the concentration of iron added to apo-BFR exceeds the amount required to saturate the ferroxidase sites. In these experiments, iron added in excess of that required to populate the ferroxidase sites is thought to bind initially to unidentified nucleation sites on the inner surface of the protein to initiate iron core formation.

Phase 3 reaction has also been studied using a pH stat and oxygen electrode (Bou-Abdallah et al. 2002). Unlike Phase 1 and 2 where proton release was much faster than oxygen consumption, Phase 3 exhibited the same rates for both proton release and oxygen consumption. Based on the stoichiometry of oxygen consumption and proton release, Phase 3 is described by the following equation.

$$4 \text{Fe(II)} + \text{O}_2 + 6 \text{H}_2\text{O} \rightarrow 4 \text{FeO(OH)(core)} + 8 \text{H}^+$$
1.7.3 Mechanism of iron core formation

The detailed mechanism by which the iron core of any member of the ferritin family forms is still not completely defined, but various models for this process have been proposed (Figure 1.6) (Lewin et al. 2005). The crystal growth mechanism of iron core formation states that small clusters of iron accumulate on the inner surface of the protein and as additional irons enter the protein, the iron cluster surface acts as additional nucleation sites for further iron core growth (Clegg et al. 1980). This model does not account for the involvement of the dinuclear iron site. On the other hand, the sequential transfer model (Figure 1.6B) involves oxidation of iron at the ferroxidase sites followed by transfer of Fe(III) to the central cavity thereby liberating the ferroxidase site to bind and oxidize additional incoming iron. In this model, the ferroxidase site acts as a catalytic site for iron oxidation. In the linked transfer model (Figure 1.6C), iron is oxidized at the ferroxidase site but is not transferred to the iron core. Growth of the iron core proceeds as additional iron ions are bound and oxidized at the surface of the iron core. The iron atoms at the ferroxidase sites cycle between reduced and oxidized states as electrons are transferred to them from the oxidizing iron core. Hence, in this model, the dinuclear iron site behaves as a cofactor instead of an active site. Other mechanisms could be envisioned as indicated in Figure 1.6D where the oxidized iron at the dinuclear iron site is unstable but is not completely transferred to the iron core. It may be partially reorganized to provide the initial nucleation site for iron core formation.
Figure 1.6. Possible mechanisms for Phase 3 iron core formation. (A) The crystal growth mechanism describes the iron core formation through the mineralization of iron at the surface of the iron core. (B) The sequential transfer model involves the oxidation and subsequent transfer of iron from the ferroxidase site to the iron core. (C) The linked transfer model is similar to the sequential transfer model but iron at the ferroxidase site is not transferred to the iron core. Instead, additional iron atoms are oxidized at the iron core surface, and the iron at the ferroxidase centre is redox cycled. (D) A combined model which describes the iron core formation process as a combination of the sequential and linked transfer models. Adapted from (Lewin et al. 2005) by permission of The Royal Society of Chemistry. (http://dx.doi.org/10.1039/b506071k)

The iron core formation mechanism in human H-chain ferritin is best described by the crystal growth and sequential transfer mechanisms. Three pathways are observed for the iron core formation reaction in human H-chain ferritin as described by the following sequence of reactions (Zhao et al. 2003).

\[
2 \text{Fe(II)} + \text{O}_2 + 4 \text{H}_2\text{O} \rightarrow 2 \text{FeOOH(core)} + \text{H}_2\text{O}_2 + 4 \text{H}^+ \\
2 \text{Fe(II)} + \text{H}_2\text{O}_2 + 2 \text{H}_2\text{O} \rightarrow 2 \text{FeOOH(core)} + 4 \text{H}^+ \\
4 \text{Fe(II)} + \text{O}_2 + 6 \text{H}_2\text{O} \rightarrow 4 \text{FeOOH(core)} + 8 \text{H}^+ 
\]
The first reaction occurs at low iron loadings and occurs at the ferroxidase site. A short lived $\mu$-1,2-peroxodiferric intermediate is observed to form at the ferroxidase site during iron oxidation by human H-chain, bullfrog M-chain, horse spleen, and *E. coli* ferritins (Zhao *et al.* 1997; Pereira *et al.* 1998; Bou-Abdallah *et al.* 2002; Zhao *et al.* 2005). The formation and decay of this intermediate is observable by multi-wavelength (i.e., rapid-scanning) stopped-flow spectroscopy by transient formation of an absorbance maximum at ~650 nm (Figure 1.7). The identity of this intermediate has been established by Mössbauer spectroscopy, resonance Raman spectroscopy and EXAFS (Moenne-Loccoz *et al.* 1998; Pereira *et al.* 1998; Hwang *et al.* 2000; Bou-Abdallah *et al.* 2002). The $\mu$-1,2-peroxodiferric intermediate is believed to decay to the more stable $\mu$-1,2-oxodiferric species with the release of hydrogen peroxide followed by hydrolysis of this resulting species and its transfer to the core at the nucleation sites. In this process, the $\mu$-1,2-oxodiferric species may be hydrolyzed prior to transferring to the core where it undergoes a second hydrolysis reaction that produces [2FeOOH] with release of two additional protons. The rapid turnover at the dinuclear iron site of H-chain ferritin has been shown to be promoted by iron in excess of the dinuclear iron site, as demonstrated by the continual production of the $\mu$-1,2-peroxodiferric intermediate at higher iron concentrations. The second pathway occurs for intermediate levels of iron loading (100-500 Fe/protein), and most importantly, consumes hydrogen peroxide produced by the first pathway. The rates at which the first and second pathways occur are similar when equal concentrations of dioxygen or hydrogen peroxide are used. The third pathway occurs for high levels of iron loading (800 Fe/protein), and is believed to occur through a mineral surface mechanism.
Figure 1.7. Detection of $\mu$-1,2-peroxodiferric intermediate in human H-chain ferritin. (A) Spectra of ferritin (5.0 $\mu$M) in MOPS (100 mM, pH 7, 25 °C) after addition of FeSO$_4$ (240 $\mu$M) every 2.5 ms for the first 0.16 s and every 17.5 ms thereafter. The rise of the peroxo species up to 50 ms is shown in black and the decay up to 14 s is shown in red. (B) Rise and decay of $\mu$-1,2-peroxodiferric intermediate after addition of various amounts of FeSO$_4$ (144-2400 $\mu$M) to ferritin (3 $\mu$M) in MOPS (100 mM, pH 7.0, 25 °C). Reproduced from (Bou-Abdallah et al. 2005) and (Zhao et al. 2003) with permission of American Chemical Society.
The iron core formation mechanism in BFR is best described by the crystal growth and linked transfer mechanisms. Oxidation of iron at the dinuclear site of BFR is thought to result in the formation of a $\mu$-1,2-oxodiferric species. The $\mu$-1,2-peroxodiferric intermediate has not been detected for BFR by stopped-flow kinetics even though iron oxidation at the ferroxidase site is expected to occur through similar intermediates (Le Brun et al. 1993b). Failure to detect this intermediate may be attributable to its highly transient nature and/or to a very low molar absorptivity that makes detection more difficult than is the case for mammalian H-chains. Interestingly, the crystal structure of BFR from *D. desulfuricans* exhibited bridging electron density located at the dinuclear iron site on the side opposite the histidyl ligands (Macedo et al. 2003). The source of this electron density could not be determined but its presence is compatible with the presence of oxygen atoms of terminal water molecules, a peroxo intermediate, a bridging oxo or hydroxo species, or a mixture of such species. Four iron atoms are oxidized for every dioxygen consumed as anticipated for the reduction of dioxygen to water (Yang et al. 2000; Bou-Abdallah et al. 2002). This stoichiometry is thought to result from the concerted reaction at two dinuclear iron sites. The first ferroxidase site reduces dioxygen to hydrogen peroxide which is subsequently reduced to water at another ferroxidase site. The addition of the following two reactions gives the net reaction for Phase 2.

$$[\text{Fe(II)}_2\text{P}]^Z + \text{O}_2 + \text{H}_2\text{O} \rightarrow [\text{Fe(III)}_2\text{O-P}]^Z + \text{H}_2\text{O}_2$$  
$$[\text{Fe(II)}_2\text{P}]^Z + \text{H}_2\text{O}_2 \rightarrow [\text{Fe(III)}_2\text{O-P}]^Z + \text{H}_2\text{O}$$

The detoxification properties of BFR arise from its ability to oxidize iron preferentially with hydrogen peroxide rather than dioxygen, thereby preventing Fenton chemistry.

When BFR with iron filled ferroxidase sites was allowed to rest for 24 hours, subsequent
addition of iron resulted in observation of Phase 3 only (Le Brun et al. 1993b). Therefore, iron at the ferroxidase site is thought to constitute a stable cofactor, and continual formation and destruction of the ferroxidase site during enzymatic turnover does not occur as in human H-chain ferritin. The rate of iron core formation depends on the amount of iron already present, which supports the crystal growth mechanism. However, the ferroxidase sites are required for iron core formation (Baaghil et al. 2003). Mutation or inhibition of the ferroxidase activity with zinc severely retards iron core formation. Figure 1.8 shows the catalytic cycle of the linked transfer model which accounts for the requirement of the ferroxidase sites, the non-regenerative nature of the ferroxidase sites, as well as the change in rates associated with the iron core size.

As isolated, the iron core of non-recombinant BFR also contains phosphate, and the amount of phosphate present in the iron core of BFR is greater than that observed in other ferritins. For example, iron to phosphate ratios of ~10 and 1.4-2.2 are observed in horse spleen ferritin and BFR respectively. The oxidation of iron in vitro in the presence of phosphate allows the incorporation of phosphate into the iron core at levels found in native cores and changes the chemical nature of the core. The presence of phosphate does not significantly affect the rate of Phase 2 but increases the rate of the Phase 3 reaction up to 5-fold (Aitken-Rogers et al. 2004). Phosphate is believed to play a role in iron binding and electron transfer at the iron core as shown by studies with A. vinelandii BFR (Watt et al. 1992).
Figure 1.8. Model for Phase 3 catalytic cycle of iron core formation in BFR. (A) Iron enters the central cavity through unknown channels. (B) As incoming iron is oxidized at the iron core surface, electrons are shuttled to the ferroxidase site. (C) The irons at the ferroxidase site are returned to the reduced state by the electrons from the oxidation of the iron at the core. (D) The irons at the ferroxidase site are oxidized by either oxygen or hydrogen peroxide. Reproduced from (Lewin et al. 2005) with permission of The Royal Society of Chemistry. (http://dx.doi.org/10.1039/b506071k)

1.8 Subunit dimer variant

Examination of the crystallographically defined structures of BFR reveals various inter-subunit contacts stabilizing the 24-meric structure. In particular, Moore, Le Brun, and co-workers, noted that Glu128 and Glu135 of one subunit stabilize oligomerization by salt bridge formation with the Arg61 and N-terminal amine of an adjacent subunit (Figure 1.9). The replacement of these glutamate residues with alanine or arginine in *Rhodobacter capsulatus* (*R. capsulatus*) BFR disrupts these interactions and prevents
formation of the 24-mer (Kilic et al. 2003). Instead, this double variant is able to form only dimers (Figure 1.10) which were found to be monodisperse even at concentrations of 160 μM (Spiro et al. 1999). The thermodynamic stability of the BFR 24-mer can be defined in terms of a $T_m$, $[\text{Urea}]_{50\%}$, and $[\text{Gnd.HCl}]_{50\%}$ of 73 °C, ~8 M, and 4.3 M respectively whereas the dimer is found to be somewhat stable (i.e., $T_m$, $[\text{Urea}]_{50\%}$, and $[\text{Gnd.HCl}]_{50\%}$ of 43 °C, ~3.2 M and 1.8 M, respectively) (Kilic et al. 2003). Nevertheless, the stability of the dimer is sufficiently great that it is amenable to a wide variety of physical, spectroscopic, and kinetic experimental approaches. Notably, this dimer possess two dinuclear iron centres and binds one heme prosthetic group.

Figure 1.9. Inter-subunit interactions stabilizing the oligomeric structure of BFR. The side chains of Glu128 and Glu135 interact with Arg61 and the amine of Met1 respectively. Each subunit exhibits two such sites of interaction with two other subunits. A total of 24 sites are present where Glu128/Glu135 of one subunit is interacting with Arg61/Met1 of another. Figure generated from PDB ID: 1BFR.
The Glu128Arg/Glu135Arg substitutions were also introduced into *E. coli* BFR to produce an assembly-deficient variant that is able to form dimeric assemblies only (Malone *et al.* 2004). Preliminary studies with this species of this variant indicate that it is able to bind and oxidize iron, i.e., that it retains ferroxidase activity. Gel filtration and sedimentation equilibrium studies verify that the oxidized iron atoms remain bound to the protein following oxidation and that the protein is dimeric. The maximum number of iron atoms that the subunit dimer variants of *E. coli* and *R. capsulatus* BFR can bind before precipitating is ~9 and ~15 respectively (100 mM MOPS, 25 °C, pH 7). The ability of *R. capsulatus* BFR to bind greater amounts of iron than *E. coli* BFR while maintaining solubility presumably results from its greater content of negatively charged residues at
the presumed nucleation surface. In view of the structural and functional integrity of the Glu128Arg/Glu135Arg BFR variants from both bacterial species, this construct can be regarded as the minimal functional unit of BFR, and it is generally referred to in this dissertation and elsewhere as the “subunit dimer variant”.

1.9 Goals of current study

The BFR subunit dimer variant provides an interesting model system for the study of BFR that permits experimental evaluation of mechanistic questions that are not readily addressed through study of the native protein. For example, the subunit dimer variant lacks inter-subunit channels that are characteristic of the wild-type 24-mer, so some mechanistic issues related to the role of these channels in reaction of the protein with iron can be evaluated with the variant. In addition, the surface of the protein that is normally exposed to the central cavity of ferritin (the “inner surface”) is exposed to the solvent. As a result, the edge of the heme prosthetic group that is not normally exposed to bulk solvent is accessible to exogenous reagents. To define better the extent to which the subunit dimer variant of *E. coli* BFR is a legitimate functional model for the wild-type protein, the current studies focussed initially on crystallographic characterization of the three-dimensional structure of this variant and on characterizing the kinetics and mechanism by which it reacts with iron. These studies were followed by related characterization of a variant of the subunit dimer in which the amino acid residues that contribute ligands to the dinuclear iron centre were replaced with the residues found at the dinuclear iron centre of the human H-chain.
CHAPTER II – MATERIALS AND METHODS

2.1 Molecular biology

The protein sample used for the determination of the subunit dimer crystal structure was prepared with the following expression system. The Glu128Arg/Glu135Arg assembly variant of E. coli BFR was expressed in E. coli MAK96 (BL21(DE3) bfr::kan) (Kilic et al. 2003) transformed with the pALN18 plasmid (pET21a containing E. coli Glu128Arg/Glu135Arg bfr gene provided by Prof. Geoffrey Moore) (Malone et al. 2004). The plasmid map and DNA sequence of the expressed protein are shown in Figures 2.1 and 2.2.

Kinetic measurements and other experiments were performed with the Glu128Arg/Glu135Arg BFR variant expressed from a pET32b+ plasmid (Novagen) following insertion of the gene encoding the Glu128Arg/Glu135Arg variant of BFR by polymerase chain reaction (PCR) integration (Geiser et al. 2001) (Figure A1). The bfr gene was amplified from the pALN18 plasmid using the TEV site primer pair (Table 2.1). The resulting PCR product was purified with a QIAquick PCR purification kit (Qiagen) before using it as a megaprimer for the amplification of a modified pET32b+ plasmid with PfuTurbo DNA polymerase (Stratagene). This plasmid (provided by Dr. Susanne Ludwiczek) contains a coding sequence for the tobacco etch virus (TEV) cleavage site (ENLYFQM) introduced immediately after the sequence encoding the S-tag (KETAAAKFERQHMDS), with cleavage occurring between Q and M (Kapust et al. 2001; Kapust et al. 2002). The resulting BFR protein is fused with a cleavable S-tag and His-tag.
Figure 2.1. Functional map of the 5.993 kbp plasmids pALN18 and pALN1. Plasmids pALN18 and pALN1 are identical except pALN18 contain the mutations which result in the Glu128Arg/Glu135Arg substitutions. Restriction sites of the multiple cloning site and genes encoded in the plasmid are indicated in the figure. Restriction sites with one and two cut sites are blue and red respectively.
Figure 2.2. DNA sequence of subunit dimer and 24-mer BFR. The differences between the DNA sequences of the subunit dimer and the 24-mer BFR are underlined. The subunit dimer variant is expressed with arginines at amino acid positions 128 and 135. The 24-mer is expressed with glutamates at positions 128 and 135. Several restriction sites are indicated.

The bfr gene was integrated into the plasmid such that the N-terminal methionyl residue of BFR is located at the end of the TEV cleavage site so that it remains attached to the protein following cleavage with TEV protease. The plasmid map and DNA sequence are shown in Figures 2.3 and 2.4. Insertion of the bfr gene into the plasmid was confirmed by DNA sequencing (BC Centre for Excellence in HIV/AIDS). Site-directed mutagenesis
was performed with a QuikChange Site-Directed Mutagenesis Kit (Stratagene) and the primer pairs in Table 2.1. The validity of all mutant genes produced was verified by DNA sequence analysis. The TEV protease variant His-TEV(S219V)-Arg expressed from the plasmid pRK793 and purified as described by Kapust et al. (2001) was the kind gift of Dr. Susanne Ludwiczek.

Table 2.1. Primers used for amplification and mutagenesis of BFR subunit dimer variants.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Forward Primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reverse Primer&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu47Gln&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’-GTG GAG TAT CAT CAG TCC ATT GAT GAG ATG AAA CAC GCC G-3’</td>
<td>5’-C GGC GTG TTT CAT CTC ATC AAT GGA CTG ATG ATA CTC CAC-3’;</td>
</tr>
<tr>
<td>Asp50Asn&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’-CC ATT AAT GAG ATG AAA CAC GCC GAT C-3’</td>
<td>5’-G ATC GGC GTG TTT CAT CTC ATT AAT GG-3’;</td>
</tr>
<tr>
<td>Glu47Gln/Asp50Asn&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5’-GTG GAG TAT CAT CAG TCC ATT AAT GAG ATG AAA CAC GCC GAT CG-3’</td>
<td>5’-CG ATC GGC GTG TTT CAT CTC ATG AAT GGA CTG ATG ATA CTC CAC-3’;</td>
</tr>
<tr>
<td>Asp126Asn</td>
<td>5’-GAA ATT TTG CGT AAT GAA CGA GGC C-3’</td>
<td>5’-G GCC TCG TTC ATT ACG CAA AAT TTC-3’;</td>
</tr>
<tr>
<td>Glu127Gln/His130Ala</td>
<td>5’-CGT GAT CAA CGA GGC GCT ATC GAC TGG CTG CGA ACG GAA CTT G-3’</td>
<td>5’-C AAG TTC CGT TCG CAG CCA GTC GAT AGC GCC TCG TTG ATC ACG-3’</td>
</tr>
<tr>
<td>TEV site</td>
<td>5’-G GGA CGC GAG AAC CTC TAC TTT CAG ATG AAA GGT GAT ACT AAA G-3’</td>
<td>5’-GCTAGTTATTCGCTACGGCGG-3’ (T7 reverse)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Underlined nucleotides indicate sites of mutations.
<sup>b</sup> Indicated primers are identical in Table 2.2.
Figure 2.3. Functional map of the 6.501 kbp plasmid pET32b-BFR. Genes encoded in the plasmid are indicated in the figure. Restriction sites with one and two cut sites are blue and red respectively.
Figure 2.4. DNA sequence of S-tagged and His-tagged subunit dimer in pET32b-BFR. The S-tag, His-tag, TEV cleavage site and several restriction sites are indicated.

The 24-mer wild-type and variants of *E. coli* BFR were expressed in *E. coli* MAK96 transformed with the pALN1 plasmid (pET21a containing *E. coli* bfr gene, provided by Prof. Geoffrey Moore) (Figures 2.1, 2.2). Nucleation site and iron entry channel mutants were made for the 24-mer using the same procedure used for the subunit dimer mutants. Site-directed mutagenesis was performed using a QuikChange Site-Directed Mutagenesis Kit and the primer pairs in Table 2.2.
Table 2.2. Primers used for amplification and mutagenesis of BFR 24-mer variants.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Forward Primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reverse Primer&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu47Gln&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5'-GTG GAG TAT CAT CAG TCC ATT GAT GAG ATG AAA CAC GCC G-3'</td>
<td>5'-C GGC GTG TTT CAT CTC ATT AAT GGA CTG ATG ATA CTC CAC-3'</td>
</tr>
<tr>
<td>Asp50Asn&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5'-CC ATT <em>AAT</em> GAG ATG AAA CAC GCC GAT C-3'</td>
<td>5'-G ATC GGC GTG TTT CAT CTC ATT AAT GG-3'</td>
</tr>
<tr>
<td>Asp126Asn</td>
<td>5'-G ATG ATA GAA ATT TTG CGT <em>AAT</em> GAA GAA GGC CAT ATC GAC-3'</td>
<td>5'-GTC GAT ATG GCC TTC TTC ATT ACG CAA AAT TTC TAT CAT C-3'</td>
</tr>
<tr>
<td>Glu47Gln/Asp50Asn&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5'-GTG GAG TAT CAT CAG TCC ATT AAT GAG ATG AAA CAC GCC GAT CG-3'</td>
<td>5'-CG ATC GGC GTG TTT CAT CTC ATT <em>AAT</em> GGA <em>CTG</em> ATG ATA CTC CAC-3'</td>
</tr>
<tr>
<td>Asp96Asn</td>
<td>5'-GAT CTG GCA CTT GAG CTG AAT GGC GCG AAG AAT TTG CGT G-3'</td>
<td>5'-C ACG CAA ATT CTT CGC GCC ATT CAG CTC AAG TGC CAG ATC-3'</td>
</tr>
<tr>
<td>Asp109Phe</td>
<td>5'-G GCA ATT GGT TAT GCC TTT AGC GTT CAT GAT TAC GTC AGC-3'</td>
<td>5'-GCT GAC GTA ATC ATG AAC GCT <em>AAA</em> GGC ATA ACC AAT TGC C-3'</td>
</tr>
<tr>
<td>Asp139Phe</td>
<td>5'-GG CTG GAA ACG GAA CTT TTT CTG ATT CAG AAG ATG <em>GGC</em> C-3'</td>
<td>5'-G GCC CAT CTT CTG AAT CAG <em>AAA</em> AAG TTC CTG TTC CAG CC-3'</td>
</tr>
<tr>
<td>Asn148Phe</td>
<td>5'-G GAG ATG GGC CTG CAA TTT TAT CTA GCA CAG ATC CGC-3'</td>
<td>5'-GCG GAT CTG TGC TTG CAG ATA <em>AAA</em> TTG CAG GCC CAT CTT C-3'</td>
</tr>
<tr>
<td>Asp109Lys</td>
<td>5'-G GCA ATT GGT TAT GCC <em>AAA</em> AGC GTC CAT GAT TAC GTC AGC CG-3'</td>
<td>5'-CG GCT GAC GTA ATC ATG GAC GCT <em>TTT</em> GCC ATA ACC AAT TGC C-3'</td>
</tr>
<tr>
<td>Asn148Lys</td>
<td>5'-G GAG ATG GGC CTG CAA <em>AAA</em> TAT CTG CAG GCA CAG ATC CGC-3'</td>
<td>5'-GCG GAT CTG TGC CTG CAG ATA <em>TTT</em> TTG CAG GCC CAT CTT C-3'</td>
</tr>
</tbody>
</table>

<sup>a</sup> Underlined nucleotides indicate the mutations.

<sup>b</sup> Indicated primers are identical in Table 2.1.
2.2 Expression and purification of BFR

2.2.1 Subunit dimer and subunit dimer variants

The protein sample used for the determination of the subunit dimer crystal structure was prepared with the following method. Overnight starter cultures of *E. coli* MAK96 cells containing pALN18 were grown in Luria Bertani media (10 g/L tryptone, 5 g/L NaCl, 5 g/L yeast extract) containing ampicilin (0.1 mg/mL) and kanamycin (0.05 mg/mL). Two-liter flasks containing Luria Bertani media (1 L) and ampicilin (0.1 mg/mL) were inoculated with the starter culture and grown at 37 ºC with 250 RPM shaking. Expression of BFR was induced with isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM; Bioshop Canada) when OD<sub>600</sub> = 1. Cells were harvested by centrifugation (15 minutes, 7100 g) 10-15 hours after induction. The cell pellet was washed with and resuspended in potassium phosphate buffer (50 mM, pH 7.2). Several freeze thaw cycles were used to lyse the cells after 1-3 hours of incubation with phenylmethanesulphonylfluoride (PMSF; 1 mM; Sigma-Aldrich) and lysozyme (1.25 mg/mL; Sigma). After lysis, deoxyribonuclease I (DNase I; 5 μg/mL; Sigma-Aldrich) and ribonuclease A (RNase A; 2 μg/mL; Sigma-Aldrich) were added to eliminate the viscous “slimy” consistency caused by released DNA. The sample was centrifuged (1 hour, 19900 g) to remove insoluble materials before applying to a DEAE-Sepharose (GE Bioscience) anion exchange column (2.75 × 10 cm) equilibrated with potassium phosphate buffer (50 mM, pH 7.2). BFR was eluted with a linear NaCl gradient (0-0.5 M) formed by mixing buffer without NaCl (300 mL) with buffer containing NaCl (0.5 M, 300 mL). Fractions containing BFR were located visually by the protein’s red colour due to the binding of heme and confirmed by SDS-PAGE. The pooled fractions were concentrated to ~5 mL.
with Amicon Ultra centrifugal filters (Millipore) before loading onto a Sephadex G-75 (GE Bioscience) gel filtration column (2.6 × 90 cm) equilibrated with potassium phosphate buffer (50 mM, pH 7.2). The protein was eluted with the same buffer using a flow rate of ~2-3 drops/min. Fractions of pure BFR as determined by SDS-PAGE were pooled (Figure A2). The protein was dialyzed with Amberlite IRC-718 (ICN Biomedicals), a metal chelating resin, contained in a separate dialysis bag to remove metals.

Protein samples used for kinetic measurements and other experiments were prepared with the following method. *E. coli* MAK96 cells transformed with the modified pET32b+ plasmids were grown and processed as described for the subunit dimer. Harvested cells were resuspended in potassium phosphate buffer (50 mM, pH 7.2) containing PMSF (1 mM), NaCl (0.5 M), and imidazole (1 mM; Fisher) prior to lysis by two passes through an Avestin Emulsiflex-C5 high pressure homogenizer at 10,000-15,000 PSI. The lysed sample was treated with DNase I and RNase A before centrifugation (1 hour, 19900 g) to remove insoluble materials. The cell-free extract was applied to a Ni²⁺-chelate column (2.75 × 2.5 cm, Chelating Sepharose FF (GE Healthcare)) and eluted with an imidazole step gradient. The column was washed with potassium phosphate buffer (50 mM, pH 7.2, 100 mL) containing NaCl (0.5 M) and imidazole (50 mM) and the protein was eluted with potassium phosphate buffer (50 mM, pH 7.2, 50 mL) containing NaCl (0.5 M) and imidazole (300 mM). Fractions containing His₆-tagged protein were incubated overnight at room temperature with TEV protease (50-100:1 BFR/TEV protease OD₂₈₀ ratio) in potassium phosphate buffer (50 mM, pH 7.2) containing NaCl (0.5 M), dithiothreitol (DTT; 1 mM), and
ethylenediaminetetraacetic acid (EDTA; 1 mM). The resulting reaction mixture was
dialyzed against potassium phosphate buffer (50 mM, pH 7.2) containing NaCl (0.5 M)
and loaded onto a Ni\textsuperscript{2+}-chelate column to remove uncleaved BFR, the cleaved tag
sequence, and the His-tagged TEV protease. The purified BFR assembly variant lacking
the N-terminal tag bound weakly to this column and was eluted with potassium
phosphate buffer (50 mM, pH 7.2, 50 mL) containing NaCl (0.5 M) and imidazole (25
mM) after washing with the same buffer containing no imidazole. Iron and other metal
ions were removed by repeated dilution and concentration by centrifugal filtration of the
protein in MES buffer (50 mM, pH 6.5) containing DTT (1 mM), EDTA (1-10 mM) and
2,2’-bipyridyl (1-5 mM). Iron removal was evidenced by the pink colour of the flow-
through exhibited when iron is bound to 2,2’-bipyridyl. Some protein precipitation is
observed but is resolubilized after buffer exchange into MES buffer (0.2 M, pH 6.5). The
addition of NaCl during buffer exchange was observed to assist in the resolubilization of
the protein.

2.2.2 Wild-type and 24-mer variants

Cells were grown and processed as described for the subunit dimer. The cell
pellet was washed with and resuspended in potassium phosphate buffer (50 mM, pH 7.2)
prior to lysis by two passes through an Avestin Emulsiflex-C5 high pressure
homogenizer. The lysed sample was treated with DNase I and RNase A. It was then
incubated at 65 °C for 10-15 minutes and placed on ice before being centrifuged to
remove insoluble materials. The cell-free extract was applied to an anion exchange
column of Sepharose-Q Fast Flow resin (20 mL; Amersham Biosciences) equilibrated
with potassium phosphate buffer (50 mM, pH 7.2) using an AKTA Purifier system (Amersham Biosciences). The column was washed with the equilibration buffer and the protein was eluted with a linear NaCl gradient (0-0.5 M). Fractions containing the protein were pooled, concentrated to >2 mL and loaded onto a gel filtration column of HiLoad Superdex-200 Preparation Grade resin (200 mL; Amersham Biosciences) equilibrated in potassium phosphate buffer (50 mM, pH 7.2). Pure fractions as determined by SDS-PAGE were pooled (Figure A2). Iron and other metals were removed by repeated dilution and concentration by centrifugal filtration of the protein in MES buffer (50 mM, pH 6.5) containing DTT (1 mM), EDTA (1-10 mM) and 2,2’-bipyridyl (1-5 mM).

2.2.3 Heme reconstitution

Less than stoichiometric amounts of heme were obtained in the overexpressed purified subunit dimer and subunit dimer variants. The concentration of protein with heme bound was determined from the absorbance at 280 nm ($3.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ subunit (Yang et al. 2000)) and 418 nm ($1.09 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (Le Brun et al. 1995)) with the molar absorptivities indicated. In order to make a homogeneous sample, heme was added to the protein to achieve full occupancy of the heme binding sites. A fresh solution of hemin chloride (1 mg/mL; Frontier Scientific, Inc.) was made by dissolving hemin chloride (100 μL/mg heme) in sodium hydroxide (0.1 M) and diluting with MES buffer (0.2 M, pH 6.5) before adding to the protein for a final 1.1:2-heme:subunit ratio. Free and adventitiously bound heme were removed by passage through a PD-10 desalting column (GE Biosciences).
2.2.4 Iron and zinc content assay

Iron content was determined using the chromogenic agents 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, monosodium salt (Ferrozine) (Stookey 1970) or 3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5’,5’’-disulfonic acid (FereneS) (Higgins 1981). FereneS is preferred over Ferrozine as it is 27% more sensitive. Protein or iron standards (150 μL) were digested with concentrated nitric acid (150 μL) at 80 ºC overnight or by boiling for 5-15 minutes in tightly capped Eppendorf tubes. Saturated sodium acetate (750 μL), freshly prepared ascorbic acid (150 μL, 50 mg/mL), and FereneS (150 μL, 10 mM) were added. The blue colour of the ferrous-FereneS complex was measured at 594 nm. Zinc content was determined using the chromogenic agent 4-(2-pyridylazo)resorcinol (PAR; Aldrich). Guanidine hydrochloride (Fischer; 500 μL, 8M) was added to protein sample (100 μL) to release zinc from BFR. PAR (100 μL, 1 mM) and HEPES (300 μL, 1 M, pH 7.4) were added before measuring the absorbance at 500 nm. The contribution from the heme was subtracted from the calculated value.

2.3 X-ray crystallographic methods

The purified Glu128Arg/Glu135Arg variant of E. coli BFR was crystallized by hanging drop vapour diffusion. Needle-shaped clusters were grown at 30 ºC by mixing equal volumes (1-3 μL) of protein (~0.2 mM in 0.2 M MES buffer pH 6.5) and precipitant solutions (30% PEG 4000, 0.2 M Li2SO4 and 0.1 M Tris-HCl buffer pH 8.5) on glass cover slips with a dried coating of Sigmacote (Sigma). The cover slips were placed over wells containing the precipitant solution (0.5 mL). A human hair taped to a pipette tip was used to lightly brush the crystals and then streaked consecutively across 6-
12 freshly made drops. The freshly made drops were similarly made by mixing equal volumes of precipitant solution with protein but PEG (20%, 4000) was used instead of PEG (30%, 4000). Brick-shaped crystals were grown using this microseeding method. Additional crystals were also made by microseeding with the brick-shaped crystals. The resulting crystals were then soaked in a series of crystallization solutions containing PEG (20%, 4000), Li$_2$SO$_4$ (0.2 M) and Tris-HCl buffer (0.1 M, pH 8.5) for 5 minutes each. These solutions contained progressively larger concentrations of ethylene glycol which increases from 5% to 30% by increments of 5%. Most of the crystals cracked from this procedure but a few appeared unaffected. The non-cracked crystals were flash frozen in liquid nitrogen and X-ray diffraction data were collected at the Stanford Synchrotron Radiation Lightsource (SSRL) (Menlo Park, CA) on beamline 1-5 using 0.979440 Å X-ray radiation and on beamline 9-2 using 1.30505 Å. These data were then indexed, integrated and scaled with the program HKL2000 (Otwinowski and Minor 1997). The structure was solved by molecular replacement with MOLREP (Vagin and Teplyakov 1997) using a pair of subunits from a structure (PDB ID: 1BCF) of wild-type E. coli BFR from which the metal ions were removed as the search model. Refinement was carried out with Refmac5 (Murshudov et al. 1997) from the CCP4 suite (1994) and with Swiss-Pdb Viewer (Guex and Peitsch 1997). The final refined model for data collected at 0.979440 Å was used to provide phases for the anomalous difference maps computed at both wavelengths. Figures were constructed with PyMOL v 1.1 (DeLano 2008), Swiss-Pdb Viewer v 3.7, and POV-Ray v 3.6.

The crystal structure of the Glu47Gln/Asp50Asn/Asp126Asn subunit dimer variant of E. coli BFR was obtained similarly to the crystal structure of the subunit dimer.
Previously, crystals of the subunit dimer grown in PEG (20%, 4000), Li$_2$SO$_4$ (0.2 M) and Tris-HCl buffer (0.1 M, pH 8.5) were soaked step-wise in increasing amounts of ethylene glycol for cryoprotection. However, this resulted in cracking of a large number of the crystals. Crystals of the Glu47Gln/Asp50Asn/Asp126Asn variant were grown with the addition of ethylene glycol (30%) in the precipitant solution to remove the soaking steps, which allowed quicker screening for suitable crystals. Crystals were flash frozen in liquid nitrogen and X-ray diffraction data were collected at the SSRL on beamline 9-2 using 1.00016 Å radiation. These data were then indexed, integrated and scaled with HKL2000. The structure was solved by molecular replacement with MOLREP using the BFR subunit dimer structure from which the zinc ions were removed as the search model. Refinement was carried out with Refmac5 and Swiss-Pdb Viewer.

2.4 Spectroscopic and kinetics methods

2.4.1 Spectroscopy of nucleation site variants

The ultraviolet-visible (UV-Vis) and circular dichroism (CD) spectra of the nucleation site subunit dimer variants were compared to determine if any structural differences possibly caused by introduction of the mutations can be detected by spectroscopy. Electronic spectra were recorded with subunit dimer (6 μM) in MES buffer (100 mM, pH 6.5) in a 1 cm path length quartz cuvette using a Varian Cary 6000i spectrophotometer. Circular dichroism spectra were recorded with subunit dimer (2.6 μM) in MES buffer (20 mM, pH 6.5) using a Jasco J-720 spectropolarimeter fitted with a circulating water bath and thermostatted cell holder (0.1 cm path length).
2.4.2 Iron oxidation kinetics

A Bio-Logic SFM-400 stopped-flow spectrometer was used for the study of Phase 2 iron oxidation kinetics. Fe(II) solutions were prepared by dissolving ferrous ammonium sulfate in HCl (6 mM) shortly before use in kinetics measurements. BFR subunit dimer (12 μM) or BFR 24-mer (1 μM) in MES buffer (0.2 M, pH 6.5) was mixed in equal volumes with iron (0, 10, 20, 30, 40, 50, 60, 80, and 100 μM) and the absorbance at 340 nm was monitored for 30 seconds. For experiments involving Zn(II), protein solutions were incubated with varying concentrations of ZnSO₄ at least 5 minutes prior to iron addition for kinetics measurements.

A Varian Cary 6000i spectrophotometer was used for the study of Phase 3 iron oxidation kinetics. Fe(II) solutions were prepared by dissolving ferrous ammonium sulfate in HCl (0.1 M) shortly before addition to apo-BFR for kinetics measurements. BFR subunit dimer (6 μM) in MES buffer (0.1 M, pH 6.5) was mixed with ferrous ammonium sulfate (12, 24, and 36 μM) and the absorbance at 340 nm was monitored for 40 minutes after 20 seconds of mixing. The dinuclear iron sites become saturated at 24 μM of iron and so Phase 3 iron oxidation kinetics are only expected to be observed for the 36 μM addition of iron. Relatively low concentrations of iron were used because high iron levels would cause the BFR subunit dimer to precipitate. For the BFR 24-mer, protein (0.5 μM) in MES buffer (0.1 M, pH 6.5) was mixed with ferrous ammonium sulfate (200 μM), and similarly, the absorbance at 340 nm was monitored for 40 minutes after 20 seconds of mixing. The concentrations of subunits in 6 μM of the subunit dimer and 0.5 μM of the 24-mer are equivalent.
An Olis RSM 1000 rapid scanning stopped-flow spectrometer was used for the study of intermediate formation in the wild-type BFR. Spectra were measured every millisecond for 4 seconds in a 2 cm cuvette after mixing BFR (18 μM) in MES buffer (0.2 M, pH 6.5) with ferrous ammonium sulfate (864 μM) freshly dissolved in HCl (6 mM). The amount of iron added was equal to the amount of iron required to fully saturate the dinuclear iron sites.

Stopped-flow measurements were performed in quintuplicate. Phase 3 measurements were performed in triplicate. Data shown are averages of experiments with error bars representing standard deviation.
3.1 Crystal structure of BFR subunit dimer

The introduction of the Glu128Arg/Glu135Arg mutations into E. coli BFR disrupts inter-subunit salt bridges and results in the formation of an assembly-defective variant. Whether or not these amino acid substitutions alter the structure of the resulting variant was not known. To evaluate the structural consequences of converting the 24-mer to a dimer, structural analysis of the dimeric variant was performed by X-ray crystallography. The initial goal of this work was to determine the structure of the derivative of the variant that is devoid of iron at the dinuclear centres but that retains bound heme.

3.1.1 Crystal growth

Crystals of the heme-bound BFR subunit dimer were obtained under various solution conditions through use of crystallization screening kits from Hampton Research and Emerald Biosystems. Early attempts at solving the structure of crystals grown in ammonium sulfate (1.5 M) and sodium citrate (0.1 M, pH 5.4) at 20 °C were unsuccessful, possibly as the result of crystal twinning. A structure was subsequently obtained from crystals grown in PEG 4000 (20-30%), Tris-HCl (0.1 M, pH 8.5) and lithium sulfate (0.2 M) (Figure 3.1). Needle-shaped crystals grown in the first round of crystallization were used for microseeding the second round of crystallization which resulted in growth of large single brick-shaped crystals with dimensions of approximately 0.1 mm × 0.1 mm × 0.3 mm.
Figure 3.1. Crystal forms of the heme-bound BFR subunit dimer. (A) Crystals grown in PEG 4K (30%), Tris-HCl (0.1 M, pH 8.5) and Li₂SO₄ (0.2 M) produced needle-shaped clusters. (B) Single brick-shaped crystals were obtained in PEG 4K (20%), Tris-HCl (0.1 M, pH 8.5) and Li₂SO₄ (0.2 M) after microseeding with crystals from (A).

3.1.2 Overview of structure

After successively soaking in increasing concentrations of ethylene glycol for cryoprotection, crystals were frozen in liquid nitrogen and sent to the SSRL for data collection. A crystal structure was solved from a dataset that was collected for a crystal that showed a clear diffraction pattern. The structure of the heme-bound Glu128Arg/Glu135Arg assembly variant BFR subunit dimer was determined by molecular replacement and refined to 1.8 Å resolution (Table 3.1). The crystal used in this work was in the P2₁2₁2₁ space group with \(a = 33.64 \text{ Å}, \ b = 91.09 \text{ Å} \) and \(c = 102.08 \text{ Å}\) and contained two subunits in the asymmetric unit (Figure 3.2). No evidence of 24-mer formation was apparent in the crystal. This result confirms that the Glu128Arg/Glu135Arg substitutions prevent BFR from forming oligomers larger than dimers. As observed in the structure of the wild-type protein, each subunit consists of four anti-parallel \(\alpha\)-helices with a fifth, shorter C-terminal \(\alpha\)-helix. Heme is bound
symmetrically as observed in the structures of wild-type BFR from *E. coli*, *D. desulfuricans*, *R. capsulatus* and *A. vinelandii* and is modeled with 50% occupancy for each of the two orientations that differ by 180° rotation about the α-γ methine carbon axis (Cobessi et al. 2002; Macedo et al. 2003; Swartz et al. 2006; van Eerde et al. 2006). After modeling all the amino acid residues into the electron density, two conformations were found for Glu96 and Glu132 in the A subunit, and for Asn17 in both subunits. In addition, each subunit possesses one metal ion bound in the ferroxidase site, one sulfate ion and two ethylene glycol molecules located nearby, all of which were modeled with 100% occupancy. Notably, one ethylene glycol molecule is located in an area of the protein related to the ferroxidase pore that has been identified in the structure of BFR from *D. desulfuricans* (Macedo et al. 2003) and the other is located near an opening at what would be the inner core surface in the wild-type protein.

### 3.1.3 Dinuclear iron site

Electron density representing the presence of metal ions was observed in the dinuclear iron site of both subunits in a position that is consistent with coordination of a metal ion by Glu18, His54, Glu51, and Glu127 in a tetrahedral geometry. This density was modeled as a Zn(II) ion and metal ligand bonds were not restrained. The presence of zinc was later confirmed by a PAR spectrophotometric zinc assay of the protein sample used for crystallization. Further evidence that this electron density is attributable to zinc at the dinuclear iron site was provided by anomalous scattering measurements of the metal when shifting the incident X-ray wavelength.
Table 3.1. Crystallographic data collection and refinement statistics of subunit dimer variant

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¹Numbers in parentheses correspond to the highest resolution shell.
Figure 3.2. Ribbon diagram of the Glu128Arg/Glu135Arg variant of *E. coli* BFR. The ethylene glycol molecules, zinc and heme are coloured yellow, magenta and red, respectively. The heme group is located between the two subunits. The model is viewed from what would be the outer surface of the 24-mer.

Anomalous scattering at the metal site decreases when compared between 0.97944 Å, which is above the K-edge of zinc, to 1.30505 Å, immediately below the K-edge of zinc (Table 3.1, Figure 3.3). Anomalous scattering would not decrease but increase if Mn, Fe, Co, Ni or Cu were located at the site in high occupancy, because both wavelengths used are above the K-edges of these metals. In agreement, an increase of anomalous scattering is seen for the heme iron and Met52 sulfur when shifting to the longer wavelength. (Figure 3.3). The peak heights at the metal sites in the anomalous difference maps were scaled by comparison to the peak height at the heme iron to account for the variation in the incident X-ray intensity at different wavelengths (Table 3.2). The ratio of
the peak heights between the heme iron and metal ion agree well with the theoretical ratio calculated for the metal being zinc.

Figure 3.3. Anomalous scattering from the heme iron and partially occupied dinuclear iron sites. Anomalous difference maps contoured at 5σ computed from data collected with incident X-ray (A) \( \lambda = 0.9794 \) Å and (B) \( \lambda = 1.3050 \) Å. The increase in peak height observed at the heme-iron for data collected at the longer X-ray wavelength is expected for iron. The decrease in peak heights observed at the dinuclear iron sites for data collected at longer X-ray wavelength indicates the presence of zinc.
Table 3.2. Peak height ratios of anomalous scattering at metal site and heme iron at different wavelengths.

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<th>Wavelength (Å)</th>
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<tr>
<td>Subunit A metal / Heme-Fe</td>
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<tr>
<td>Zn / Heme (theoretical)</td>
<td>1.66 0.21</td>
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</table>

As described in Section 2.2.1, the preparation of the BFR subunit dimer variant used in the crystallization studies had been exchanged repeatedly against buffer containing a dialysis bag of chelating resin to remove metal ions. Thus the mechanistic implications of the presence of Zn(II) in only one part of the ferroxidase site are uncertain though it does seem apparent that the two metal ion binding sites are inequivalent insofar as zinc binding is concerned. The notable result here is that Zn(II) has been found at the ferroxidase site (full occupancy by zinc or as a mixture with iron), as it was in recent reports of the structure of *E. coli* 24-mer protein (van Eerde et al. 2006; Willies et al. 2009). This observation provides strong evidence in favour of the suggestion that Zn(II) inhibits BFR by binding at its ferroxidase centre.

The metal ion binding site for the other half of each dinuclear site consists of the ligands Glu94, His130, Glu51, and Glu127. This site exhibits no occupancy by metal ions in the current data set (Figure 3.4). Interestingly, His130 is rotated away from the ferroxidase site as observed previously in the structures of a uranyl derivative of *E. coli* BFR (Frolov and Kalb 2001) and *A. vinelandii* BFR with a partially occupied ferroxidase
site (Liu et al. 2004; Swartz et al. 2006). His130 also has a high B-factor in a previously reported crystal structure of *E. coli* BFR, indicating flexibility in this residue (van Eerde et al. 2006). Electron density adjacent to the dinuclear iron site and not connected to any protein chains is modelled as ethylene glycol. Crystals were soaked in solutions containing ethylene glycol prior to freezing for cryoprotection and ethylene glycol presumably filled the pockets next to the dinuclear iron site.

Figure 3.4. Images of the partially occupied dinuclear iron site. (A) $2F_o - F_c$ representative electron density of the dinuclear iron site contoured at 1σ (blue) and 5σ (green). (B) The metal ion (magenta) and ethylene glycol (yellow) are highlighted. His130 is directed away from the vacant site where the iron is normally coordinated by His130.
3.1.4 Discovery of ethylene glycol in structure

Two ethylene glycol molecules are modelled into electron density found near the dinuclear iron site. For each subunit, one ethylene glycol molecule is located between the bound metal ion and the “outer surface” and the other ethylene glycol molecule is located between the empty site and the “inner surface” (Figure 3.5). The “outer surface” and “inner surface” refer to the surfaces of the protein which normally face the exterior and interior, respectively, of the 24-mer spherical shell-like structure (Figure 3.5A). The “outer” ethylene glycol is bound in a pocket that is surrounded by the residues Leu14, Asn17, Glu18, Tyr58, Gly97, Glu127, and either waters 10 and 16 in subunit A or waters 28 and 78 in subunit B. One end of this ethylene glycol molecule appears to form an H-bond with Tyr58 and Glu127, and the other appears to form H-bonds with Glu127, Asn17, and Glu18 as well as two water molecules. Viewed from the outer surface, the ethylene glycol can be seen to fill a pocket that opens to the ferroxidase pore (Figure 3.5B). The ferroxidase pore is lined with Asn17 which has multiple conformations in the current structure. The position of the pore opening changes depending on the conformation of Asn17.

One hydroxyl end of the “inner” ethylene glycol is buried in the empty dinuclear iron site and is bound by Glu51, Glu94 and Glu127 while the other hydroxyl group is not bound by the protein but points towards the opening that is surrounded by acidic residues Glu47, Asp50, and Asp126. Interestingly, the positions of Glu47 and His46, adjacent to the ferroxidase site, and His130, on the inner surface of the protein (Figure 3.5C, D), differ from their positions in the structure of *E. coli* BFR with a fully occupied ferroxidase site (Frolov et al. 1994; Dautant et al. 1998; van Eerde et al. 2006; Willies et
al. 2009). Viewed from the “inner surface”, the ethylene glycol can be seen to fill a pocket that leads to the position in the dinuclear site that is normally occupied by His130 when a metal ion is present (Figure 3.5D). The proximity of Glu47, Asp50, and Asp126 to the ferroxidase site and their negative charge suggest the possibility of their involvement in iron core nucleation.

Figure 3.5. Molecular surfaces showing the ferroxidase pore openings at the inner and outer surface. (A) Side view showing the relative positions of the ethylene glycol molecules and metal ion inside the protein. (B) Outer surface of BFR subunit dimer showing ethylene glycol molecule coloured yellow in outer pore. (C) Inner surface of wild-type BFR showing previously observed conformations of His46, Glu47 and His130 in the “closed” state. (D) Inner surface of BFR subunit dimer showing ethylene glycol molecule in inner pore and alternate conformations of His46, Glu47 and His130 in the “open” state.
3.2 Kinetics of iron oxidation by BFR subunit dimer

Concurrently with obtaining the crystal structure of the BFR subunit dimer, a new construct was made by attaching a cleavable poly-histidine tag to BFR to facilitate protein purification. As well, a TEV protease recognition site was placed before the N-terminal methionyl residue such that cleavage by the TEV protease would result in the full length BFR subunit dimer protein without extra or missing amino acids. The following results described for the BFR subunit dimer and variants of this protein were obtained with protein prepared from the new construct. The observation of zinc in the crystal structure also led to modification of the procedure for removal of metal ions to assure that all metal ions were removed. The effectiveness of this method was verified by spectrophotometric iron and zinc content assays.

3.2.1 Phase 2 of iron oxidation by BFR subunit dimer

On addition of Fe(II) to the subunit dimer variant, a biphasic increase in absorbance at 340 nm is observed. The initial burst phase of oxidation as monitored at this wavelength, the so-called Phase 2, is clearly distinct from the slower Phase 3. The kinetics of Phase 2 are studied by stopped-flow mixing experiments in which equal volumes of the iron-free protein (12 μM) and Fe(II) of varying concentrations are mixed, and the resulting absorbance change is monitored at 340 nm (Figure 3.6A). As can be seen, the change in absorbance at 340 nm reaches a maximum when ~2 equivalents (24 μM) of Fe(II) are added for each BFR subunit. This amount of iron corresponds to the number of iron atoms required to occupy the dinuclear iron sites completely (Figure 3.6B). For iron loadings of 25 μM and greater, absorbance at 340 nm decreases slightly
after completion of Phase 2. As a result, a lag phase is observed prior to Phase 3. This lag phase has been observed for corresponding kinetics of wild-type BFR in the presence of phosphate (1 mM) (Aitken-Rogers et al. 2004). However, the samples used in the current experiments were repeatedly concentrated and diluted with MES buffer (0.2 M, pH 6.5) by centrifugal ultrafiltration and should not contain phosphate. These results provide the first clear demonstration that the ferroxidase centres of the subunit dimer variant exhibit activity similar to that of the wild-type 24-mer, an observation not provided by the previous kinetic study of this variant (Malone et al. 2004). Consequently, these results provide critical validation of the subunit dimer as a functional model that is relevant to the mechanisms by which the intact 24-mer wild-type protein functions.

### 3.2.2 Phase 3 of iron oxidation by BFR subunit dimer

The Phase 3 kinetics following addition of various concentrations of Fe(II) to apo-BFR subunit dimer are shown in Figure 3.7. Phase 3 iron oxidation involves oxidation of Fe(II) that remains after completion of Phase 2 and so is observed only under conditions where more Fe(II) is added than is required for full occupancy of the dinuclear sites. In this type of experiment, at iron concentrations of 12 μM and 24 μM, a large increase in absorbance occurs prior to initiation of data acquisition (~20 sec after mixing). Addition of Fe(II) at concentrations less than or equal to the amount required to fully occupy the dinuclear sites, the absorbance value remains relatively constant after the initial increase because all the iron is bound quickly and oxidized at the ferroxidase sites (Phase 2). When 36 μM of iron is added, the ferroxidase sites become saturated, and excess iron is expected to bind at nucleation sites and oxidize slowly (Phase 3). The
initial large increase in absorbance observed after addition of 36 μM iron is similar to that observed following addition of 24 μM iron, consistent with saturation of the ferroxidase sites at the lower concentration of Fe(II). After a short lag phase during which Phase 2 transitions to Phase 3 (Figure 3.7B), Phase 3 is evident from the slow, continued increase in absorbance at 340 nm.

Figure 3.6. Phase 2 iron oxidation kinetic measurements of BFR subunit dimer. (A) Fe(II) was added to final concentrations of 0, 5, 10, 15, 20, 25, 30, 40 and 50 μM to BFR subunit dimer (6 μM) in MES buffer (0.1 M, pH 6.5, 25 °C) using a stopped-flow instrument. (B) Final absorbance change after 30 seconds of iron oxidation at various ratios of Fe(II)/BFR subunit concentration. Dotted line represents concentration of iron required to fully occupy the dinuclear iron site.
Figure 3.7. Phase 3 iron oxidation kinetic measurements of BFR subunit dimer. (A) Fe(II) was added to final concentrations of 12, 24 and 36 μM to BFR subunit dimer (6 μM) in MES buffer (0.1 M, pH 6.5, 25 °C). (B) Close up of the Phase 2-Phase 3 transition for iron oxidation of 36 μM of iron.

3.3 Mutation of residues 47, 50, and 126 in BFR subunit dimer

Three acidic residues, Glu47, Asp50 and Asp126, have been noted previously to occur on the inner surface of the protein in proximity to the ferroxidase centre (Baaghil et al. 2003; Malone et al. 2004). In the structure shown in Figure 3.5D, these acidic residues
are also notable because they form a negative electrostatic surface near one of the ethylene glycol molecules that is partially exposed on the inner surface of the protein. The proximity of these residues to this bound ethylene glycol molecule reinforces the previous suggestion that this region of the protein provides a possible site for nucleation of the iron core. To evaluate this possibility, site-directed mutagenesis was used to replace Glu47, Asp50, and Asp126 with the corresponding amidated residues, individually (the Glu47Gln, Asp50Asn and Asp126Asn variants) and in combination with each other (Glu47Gln/Asp50Asn/Asp126Asn or the QNN variant), for kinetic analysis.

### 3.3.1 UV-Vis and circular dichroism spectroscopy

The far-UV CD spectra of the BFR subunit dimer and its Glu47Gln, Asp50Asn and Asp126Asn variants are essentially identical to each other (Figure 3.8A). These spectra are all characteristic of spectra expected for proteins with substantial \( \alpha \)-helical content. Similarly, the electronic absorption spectra of these proteins exhibit Soret maxima at 418 nm and weak maxima at 650 nm and 737 nm that are indicative of bis-methionine heme ligation (Figure 3.8B)(Andrews et al. 1993; Andrews et al. 1995). These spectroscopic data provide evidence that the heme binding sites, the high content of helical structure, and the three-dimensional structure of the subunit dimer in general are not perturbed to any significant extent by the replacement of the surface carboxylates by amides.
Figure 3.8. Comparison of far-UV circular dichroism and UV-Vis spectra of BFR subunit dimer nucleation site variants. (A) CD spectra of BFR subunit dimer (---), subunit dimer Glu47Gln (-----), subunit dimer Asp50Asn (---), subunit dimer Asp126Asn (...), and subunit dimer Glu47Gln/Asp50Asn/Asp126Asn (---) in MES buffer (20 mM, pH 6.5). (B) UV-Vis spectra of BFR subunit dimer (---), subunit dimer Glu47Gln (-----), subunit dimer Asp50Asn (---), subunit dimer Asp126Asn (---), and subunit dimer Glu47Gln/Asp50Asn/Asp126Asn (---) in MES buffer (100 mM, pH 6.5).
3.3.2 Ferroxidase Phase 2 kinetics of BFR subunit dimer nucleation site variants

Stopped-flow kinetic measurements were used to evaluate the effect of neutralizing each of the negative charges of the acidic residues adjacent to the ethylene glycol molecule on the inner surface of the BFR subunit dimer by analysis of the behaviour of the Glu47Gln, Asp50Asn and Asp126Asn variants during Phase 2 of the ferroxidase reaction. These experiments were performed as described above for the wild-type subunit dimer, and so these variants were mixed with varying excess concentrations of Fe(II) and iron oxidation was observed by measuring the changes at 340 nm (Figure 3.9).

The Asp50Asn and Asp126Asn variants exhibit kinetics of Phase 2 activity that are slightly different to those of the wild-type subunit dimer (Figure 3.10). As shown before, the wild-type protein reaches saturation of the Phase 2 activity at ~2 Fe/subunit, which is the amount of iron required to fill the ferroxidase sites (Figure 3.10A). For the Asp50Asn variant, the plateau in activity reached after saturation of the Phase 2 activity is not as clearly defined while this plateau is clearly defined for the Asp126Asn variant (Figure 3.10A).

The Glu47Gln variant exhibits greatly diminished Phase 2 activity (Figure 3.10B). A possible reason for the greater influence exhibited by replacement of Glu47 than the other acidic residues of this group may be the shorter distance between Glu47 and FE2 (6.3 Å as opposed to 7.6 Å and 8.0 Å for Asp50 and Asp126, respectively, as measured from the side chain carboxyl carbon in the structure of van Eerde et al. (2006)). The Glu47Gln/Asp50Asn/Asp126Asn (QNN) variant exhibits the greatest alteration in behaviour in that it exhibits virtually no Phase 2 activity (Figure 3.10B).
Figure 3.9. Phase 2 iron oxidation kinetics of BFR subunit dimer nucleation site variants. Fe(II) was added to final concentrations of 0, 5, 10, 15, 20, 25, 30, 40 and 50 μM to 6 μM (A) BFR subunit dimer, (B) subunit dimer Glu47Gln, (C) subunit dimer Asp50Asn, (D) subunit dimer Asp126Asn and (E) subunit dimer Glu47Gln/Asp50Asn/Asp126Asn in MES buffer (0.1 M, pH 6.5, 25 °C) using a stopped-flow instrument.
Figure 3.10. Phase 2 final absorbances and rates for BFR subunit dimer nucleation site variants. (A) Final absorbance change after 30 seconds of iron oxidation and (B) initial rates of iron oxidation after addition of ferrous ammonium sulfate (0, 5, 10, 15, 20, 25, 30, 40 and 50 μM) to 6 μM (■) BFR subunit dimer, (●) subunit dimer Glu47Gln, (▲) subunit dimer Asp50Asn, (▼) subunit dimer Asp126Asn, and (♦) subunit dimer Glu47Gln/Asp50Asn/Asp126Asn in MES buffer (0.1 M, pH 6.5, 25 °C).
3.3.3 Ferroxidase Phase 3 kinetics of BFR subunit dimer nucleation site variants

The kinetics of Phase 3 iron oxidation by the dimeric variant of BFR was compared with that of the amidated variants studied above (Figure 3.11). Under the conditions of these experiments, full occupancy of the dinuclear iron sites is expected to occur at an iron concentration of 24 μM. The Asp50Asn and Asp126Asn variants exhibited similar iron oxidation activity up to 24 μM of iron added and diminished Phase 3 activity relative to the wild-type dimeric BFR variant (compare Figure 3.11A with Figure 3.11C, D). As observed for the Phase 2 kinetics, the Glu47Gln and the QNN variant exhibit reduced activity at all iron concentrations, but their activity is greater than that observed for iron autooxidation alone. Once again, the QNN variant exhibits the most dramatically diminished ferroxidase activity Fe(II) (Figure 3.11B, E). The BFR subunit dimer, Glu47Gln variant, Asp50Asn variant, Asp126Asn variant, and QNN variant have Phase 3 rates of 0.019(2), 0.0107(8), 0.0035(3), 0.0097(4), and 0.011(3) × 10^{-3} AU/sec respectively (Figure 3.12). These values were determined for the data collected after the first 10 minutes of the reaction to avoid contributions from Phase 2 or the lag phase.
Figure 3.11. Ferroxidase Phase 3 kinetics of BFR subunit dimer nucleation site variants. Fe(II) was added to final concentrations of 12, 24 and 36 μM to 6 μM (A) BFR subunit dimer, (B) subunit dimer Glu47Gln, (C) subunit dimer Asp50Asn, (D) subunit dimer Asp126Asn, and (E) subunit dimer Glu47Gln/Asp50Asn/Asp126Asn in MES buffer (0.1 M, pH 6.5, 25 °C). (F) Autoxidation of Fe(II) was measured in MES buffer (0.1 M, pH 6.5, 25 °C) in the absence of protein.
Figure 3.12. Ferroxidase Phase 3 rates of BFR subunit dimer nucleation site variants. Fe(II) (36 μM) was added to 6 μM BFR subunit dimer, subunit dimer Glu47Gln, subunit dimer Asp50Asn, subunit dimer Asp126Asn, and subunit dimer Glu47Gln/Asp50Asn/Asp126Asn in MES buffer (0.1 M, pH 6.5, 25 ºC). The Phase 3 rate was calculated based on the iron oxidation curve at 10 minutes to avoid contributions from Phase 2.

3.3.4 Crystal structure of BFR subunit dimer E47Q/D50N/D126N variant

Of the four nucleation site variants constructed, the crystal structure of the subunit dimer QNN variant with heme bound was obtained. The conditions for crystal growth were similar to those used for crystallization of the wild-type subunit dimer, except instead of soaking the crystals in successively greater concentrations of ethylene glycol, the crystals were grown in a solution containing ethylene glycol at the final concentration required for cryoprotection. The structure of the QNN variant containing heme was determined by molecular replacement using the structure of the subunit dimer as a model and refined to 1.66 Å resolution (Table 3.3). Under these conditions, this protein crystallizes in the space group P2_12121 with \( a = 32.8 \) Å, \( b = 91.6 \) Å and \( c = 102.6 \) Å and
contains two subunits in the asymmetric unit. All the residues were modelled into the
electron density with none of the residues in alternative conformations. The structure of
this QNN variant is remarkably similar to that of the wild-type subunit dimer structure
with an RMSD of 0.29 Å calculated over all Cα atoms.

Table 3.3. Crystallographic data collection and refinement statistics of
subunit dimer QNN variant.

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</table>

¹Numbers in parentheses correspond to the highest resolution shell.
No electron density was observed at the dinuclear iron site of the QNN variant, indicating that the revised method for metal ion removal was effective. Even though the crystals were grown in ethylene glycol, no electron density was observed corresponding to the ethylene glycol molecules observed in the structure of the wild-type BFR subunit dimer. Nevertheless, His130 is observed, again, to occur in an “open” conformation as in the structure determined for the subunit dimer variant. Comparison of all atoms of residues comprising the dinuclear iron site of the wild-type subunit dimer with that of the QNN variant subunit dimer shows slight deviations with an RMSD of 0.43 Å. The principal differences between these two structures appear to be small differences in the rotational angles of the side chains (Figure 3.13). Thus, the Glu47Gln/Asp50Asn/Asp126Asn substitutions in the subunit dimer do not perturb the structure of the ferroxidase site significantly.

Figure 3.13. Comparison of the dinuclear iron site of the BFR subunit dimer and the subunit dimer E47Q/D50N/D126N variant. The dinuclear iron site of the subunit dimer variant (blue) and the QNN variant (red) are overlapped. The dinuclear iron site of the QNN variant contains no metal ions or ethylene glycol. The zinc and ethylene glycol molecules at the active site of the wild-type subunit dimer variant are not shown.
The area of greatest significant difference between the structures of the wild-type subunit dimer and the QNN variant is the “inner” surface of the protein. In the variant structure, the side chains of Gln47, Asn50, and Asn126 have moved closer together relative to their positions in the subunit dimer variant (Figure 3.14A). Notably, the amide group of Asn126 is rotated to point towards the other two residues rather than away from them as in the structure of the wild-type subunit dimer. The opening occupied by the ethylene glycol molecule in the structure of the wild-type subunit dimer is not present in the structure of the QNN variant (Figure 3.5D, 3.14B). Presumably, amidation of the carboxyl group of these residues eliminates the normal electrostatic repulsion that prevents their side chains from approaching each other so that this opening has collapsed into a closed state. This electrostatic alteration results in hydrogen bonding of the side chains of Asn50 and Asn126 with Gln47 and of Gln47 with Glu51.
**Figure 3.14. Inner surface of the subunit dimer E47Q/D50N/D126 N variant.** (A) Superposition of the BFR subunit dimer and the subunit dimer QNN variant structures. The BFR subunit dimer is coloured in blue and the residues of interest are highlighted in cyan. The QNN variant is coloured in maroon, and the residues of interest are highlighted in red. (B) The “inner” surface of QNN variant is shown and can be seen to exhibit no opening to the dinuclear iron site. The opening in which an ethylene glycol molecule was seen is absent as the result of the E47Q/D50N/D126N substitutions.

### 3.4 Mutation of residues 47, 50, and 126 in BFR 24-mer

The Glu47Gln, Asp50Asn and Asp126Asn substitutions in the wild-type 24-mer BFR were constructed to determine what effects these residues have on ferroxidase activity of this protein relative to their effect on the ferroxidase activity of the subunit
dimer variant. Stopped-flow measurements were performed as described above to investigate the effects of removing the negative charges from Glu47, Asp50 and Asp126 on Phase 2 of iron core formation in the 24-mer (Figure 3.15). The dinuclear iron sites of wild-type BFR are saturated at 48 Fe/BFR, which is approximately the amount of iron that is required for the final absorbance change for Phase 2 to reach a plateau (Figure 3.16A). For the Asp50Asn and Asp126Asn variants, Phase 2 activity also approaches a similar plateau, indicating saturation of the dinuclear iron sites (Figure 3.16A), although the plateau is not as well defined as in the case of the wild-type 24-mer. Specifically, unlike the situation for wild-type BFR where iron oxidation appears to stop after 10 seconds, the Asp50Asn and Asp126Asn variants appear to continue oxidizing iron at a slow rate. The Glu47Gln and QNN variants do not exhibit Phase 2 saturation with a clearly defined plateau, presumably because these variants oxidize Fe(II) at a significantly reduced rate. Overall, the rate of Phase 2 Fe(II) oxidation exhibited by the Asp50Asn variant is most similar to that of the wild-type protein, the activity of the Asp126Asn variant is reduced somewhat more (Figure 3.16B), and the activities of the Glu47Gln and QNN variants are diminished substantially, as noted in the corresponding experiments with the subunit dimer variants. Interestingly, however, introduction of the Glu47Gln and QNN substitutions into the 24-mer result in much greater residual activities than do the same substitutions introduced into the subunit dimer (Figure 3.9, 3.15).
Figure 3.15. Phase 2 iron oxidation kinetic measurements of BFR 24-mer nucleation site variants. Fe(II) was added to final concentrations of 0, 5, 10, 15, 20, 25, 30, 40, and 50 μM to 0.5 μM (A) wild-type, (B) Glu47Gln, (C) Asp50Asn, (D) Asp126Asn, and (E) Glu47Gln/Asp50Asn/Asp126Asn BFR in MES buffer (0.1 M, pH 6.5, 25 °C) with a stopped-flow instrument.
Figure 3.16. Phase 2 final absorbances and rates of B FR 24-mer nucleation site variants. (A) Final absorbance changes after 30 seconds of iron oxidation and (B) initial rates of iron oxidation after addition of Fe(II) (0, 5, 10, 15, 20, 25, 30, 40, and 50 μM) to 0.5 μM (■) wild-type, (●) Glu47Gln, (▲) Asp50Asn, (▼) Asp126Asn, and (♦) Glu47Gln/Asp50Asn/Asp126Asn in MES buffer (0.1 M, pH 6.5, 25 °C).
Kinetics measurement of Phase 3 of the ferroxidase reaction catalyzed by the 24-mer nucleation site mutants produced results similar to those obtained for the corresponding variants of the subunit dimer (Figure 3.17). A greater amount of iron was added to the 24-mer than the subunit dimer for measurement of Phase 3 iron oxidation in these experiments because the 24-mer can bind more iron without precipitation than is the case for the subunit dimer. Use of greater concentrations of Fe(II) is advantageous in these experiments because more of the total absorbance change will be due to the Phase 3 nucleation activity rather than the Phase 2 ferroxidase site activity. The Glu47Gln single variant exhibited the least Phase 3 activity of the single site variants, and the QNN variant exhibited the most significantly reduced rate, which is the same pattern of reactivity observed for the corresponding substitutions in the subunit dimer. The BFR wild-type, Glu47Gln variant, Asp50Asn variant, Asp126Asn variant, and QNN variant exhibit Phase 3 rates of $0.354(6)$, $0.068(5)$, $0.086(6)$, $0.08(1)$, and $0.0196(1) \times 10^{-3}$ AU/sec respectively (Figure 3.18). The values were determined for the data collected after the first 5 minutes of reaction to avoid contributions from Phase 2 or the lag phase.
Figure 3.17. Phase 3 iron oxidation kinetic measurements of BFR 24-mer nucleation site variants. Fe(II) (200 μM) was added to 0.5 μM wild-type(●), Glu47Gln(●), Asp50Asn(●), Asp126Asn(●), and Glu47Gln/Asp50Asn/Asp126Asn(●) in MES buffer (0.1 M, pH 6.5, 25 °C).
Fe(II) (200 μM) was added to 0.5 μM wild-type, Glu47Gln, Asp50Asn, Asp126Asn, and Glu47Gln/Asp50Asn/Asp126Asn in MES buffer (0.1 M, pH 6.5, 25 °C). The Phase 3 rate was calculated based on the iron oxidation curve at 5 minutes to avoid contributions from Phase 2.

3.5 Replacement of channel residues in the BFR 24-mer

The main channel used for iron entry in human H-chain ferritin is the 3-fold channel (Treffry et al. 1993; Levi et al. 1996; Bou-Abdallah et al. 2002; Bou-Abdallah et al. 2008). Currently, however, it is not clear which channels in the structure of BFR are used for iron entry into the ferroxidase site or for entry to the inner core where the nucleation phase occurs. Two possible routes have been identified for iron to enter the ferroxidase site: the ferroxidase pore located at the outer surface and the opening located at the inner surface. The opening to the ferroxidase pore is lined by Asn17 and Leu93, with Asp96 close by. The opening at the inner surface is surrounded by Glu47, Asp50, and Asp126. Several inter-subunit channels have been proposed for iron to enter the core.
of BFR. The 3-fold channel in *E. coli* BFR is lined with the charged residues Asp109, Arg117 and Asp118 while the 4-fold channel is lined with the polar residues Asn148 and Gln151. The B-site channel is lined with Asp132, Glu135, Thr136, and Asp139 from one subunit and Asn34 and Glu66 from two other different subunits. For comparison, the human H-chain ferritin 3-fold channel is lined with charged residues Glu134 and Asp131, and the 4-fold channel of the H-chain is lined primarily with hydrophobic residues Leu165, Leu169 as well as His173.

BFR 24-mer variants with altered amino acids at the 3-fold channel, 4-fold channel, B-site channel, and ferroxidase pore were constructed with the intention of blocking these openings so the channels involved in iron transport for iron core formation could be determined. Specifically, Asp109 (3-fold), Asn148 (4-fold), Asp139 (B-site), and Asp96 (FP) were converted to asparagine or phenylalanine either to remove a negative charge that might be involved in attracting iron ions or to provide steric hindrance at the channel openings. These substitutions should have no effect on iron oxidation by the subunit dimer variant if they impede iron entry through blockage of the inter-subunit channels as the subunit dimer variant is devoid of inter-subunit channels.

Stopped-flow measurements were performed to investigate the effect of the channel substitutions on Phase 2 of the ferroxidase reaction, which occurs at the ferroxidase site (Figure 3.19). The final absorbance change for Phase 2 reaches a plateau following the saturation of the dinuclear iron sites in wild-type BFR at 48 Fe/BFR (Figure 3.20A). For all the variants tested, the Phase 2 reaction also reaches a plateau consistent with saturation of the dinuclear iron sites (Figure 3.20A). The behaviour of the Asp139Phe variant differs from that of wild-type BFR in that it exhibits a greater final
absorbance plateau value. The initial rates of Phase 2 iron oxidation are similar for wild-type BFR and the Asp109Phe/Asn148Phe variant but the Asp96Asn and Asp139Phe variants exhibit rates that are greater than that of the wild-type protein at higher concentrations of iron (Figure 3.20B).

Figure 3.19. Phase 2 iron oxidation kinetic measurements of BFR 24-mer channel variants. Fe(II) was added to final concentrations of 0, 5, 10, 15, 20, 25, 30, 40, and 50 μM to 0.5 μM (A) wild-type, (B) Asp109Phe/Asn148Phe, (C) Asp96Asn, and (D) Asp139Phe BFR in MES buffer (0.1 M, pH 6.5, 25 °C) with a stopped-flow instrument.
Figure 3.20. Phase 2 final absorbances and rates of B FR 24-mer nucleation site variants. (A) Final absorbance changes after 30 seconds of iron oxidation and (B) initial rates of iron oxidation after addition of Fe(II) (0, 5, 10, 15, 20, 25, 30, 40, and 50 μM) to 0.5 μM (-■-) wild-type, (-●-) Asp109Phe/Asn148Phe, (-▲-) Asp96Asn, and (-▼-) Asp139Phe in MES buffer (0.1 M, pH 6.5, 25 °C).
Phase 3 activities of the 24-mer channel variants were measured by observing the increase of absorbance at 340 nm after adding iron to metal free samples. The rates of this phase of iron oxidation exhibited by all of these variants were similar to the wild-type value (Figure 3.21). Specifically, the wild-type, Asp139Phe, Asp96Asn, Asp109Phe, Asn148Phe, and Asp109Phe/Asn148Phe variants have rates of 0.354(6), 0.35(2), 0.514(7), 0.443(3), 0.42(1), and $0.42(2) \times 10^{-3}$ AU/sec respectively (Figure 3.22). None of these variants exhibited a clear reduction in activity that helps discern which of these channels participates in iron influx. Surprisingly, some of the variants exhibited a slight increase in iron oxidation rate with the ferroxidase pore variant (Asp96Asn) exhibiting the largest increase.

![Figure 3.21. Phase 3 iron oxidation kinetics of BFR 24-mer channel variants.](image)

Fe(II) (200 μM) was added to 0.5 μM wild-type (▬), Asp109Phe (▬), Asn148Phe (▬), Asp139Phe (▬), Asp96Asn (▬), and Asp109Phe/Asn148Phe (▬) BFR in MES buffer (0.1 M, pH 6.5, 25 °C).
Figure 3.22. Phase 3 iron oxidation rates of BFR 24-mer channel variants. Fe(II) (200 μM) is added to 0.5 μM wild-type, Asp139Phe, Asp96Asn, Asp109Phe, Asn148Phe, and Asp109Phe/Asn148Phe in MES buffer (0.1 M, pH 6.5, 25 ºC). The Phase 3 rate is calculated based on the iron oxidation curve at 5 minutes to avoid contributions from Phase 2.

3.6 Zinc inhibition of iron oxidation by BFR subunit dimer

Zinc has been shown previously to inhibit the ferroxidase activity of wild-type BFR. Though the mechanism of this inhibition has been proposed to involve binding of Zn(II) to the ferroxidase sites (Le Brun et al. 1995; Le Brun et al. 1996; Yang et al. 2000), there is no direct evidence for this conclusion. Thus, it remains possible that Zn(II) inhibits 24-mer BFR by binding to the channels located between protein subunits, thereby preventing access of Fe(II) to the central cavity. As the latter mechanism is not possible with the subunit dimer variant used in the current work, the effect of Zn(II) on the ferroxidase activity of the subunit dimer variant was evaluated by stopped-flow experiments in which increasing concentrations of Zn(II) were added to BFR prior to
mixing with Fe(II). Addition of Zn(II) to the apo-BFR subunit dimer inhibits oxidation of Fe(II) in a manner that is not linearly dependent on Zn(II) concentration and that is essentially complete following the addition of two equivalents of Zn(II) for each BFR subunit (Figure 3.23). Thus, the effect of Zn(II) on the kinetics of Fe(II) oxidation does not require the presence of channels that are present in the structure of the wild-type 24-mer protein, consistent with Zn(II) inhibiting Fe(II) oxidation by binding to the ferroxidase centres.

Figure 3.23. Inhibition of Phase 2 iron oxidation in BFR subunit dimer by zinc. (A) Inhibition of Phase 2 iron oxidation by various zinc concentrations was measured by stopped-flow spectroscopy at 25 °C. Fe(II) (25 μM) was added to BFR subunit dimer (6 μM) in MES buffer (0.1 M, pH 6.5) containing Zn(II) (0, 6, 12, 18 or 24 μM). (B) The dependence of the initial rates of iron oxidation by the BFR subunit dimer following addition of varying concentrations of zinc. (C) The final absorbance at completion of Phase 2 by the BFR subunit dimer in the presence of varying concentrations of zinc.
3.7 Creating a human-like ferroxidase site in BFR subunit dimer

Comparison of the dinuclear iron sites in BFR and human H-chain ferritin reveal a difference of two amino acids that provide ligands to the bound iron. Specifically, Glu127 and His130 of *E. coli* BFR are replaced by a glutamine and glutamate respectively in the human protein. However, alignment of the BFR and human H-chain ferritin structures suggests that three residues at the BFR ferroxidase site require alterations for conversion of the active site of BFR to mimic the active site in the human protein. The glutamyl residue that replaces His130 in the human H-chain actually occupies the position of Asp50 in BFR. Therefore, to recreate the human site in BFR, His130 was replaced with alanine to remove any interference that might result from the presence of His at position 130, and Asp50 was replaced with glutamate. The BFR subunit dimer Asp50Glu/Glu127Gln/His130Ala variant (“human-like”) was prepared to determine whether or not changing these residues would be sufficient to convert the mechanism of the BFR ferroxidase mechanism equivalent to that of the human H-chain.

Consequently, the ferroxidase reaction of the “human-like” BFR was measured by observing the increase in absorbance at 340 nm after iron addition as reported above. Notably, the ferroxidase activity of this variant is reduced relative to that of the wild-type BFR subunit dimer and a large increase in absorbance was not observed after mixing. It was apparent that the “human-like” variant does not exhibit the Phase 2 that is characteristic of the BFR subunit dimer (Figure 3.24).
Figure 3.24. Kinetic measurements of iron oxidation in human-like BFR subunit dimer variant. Fe(II) was added to final concentrations of 12 μM, 24 μM and 36 μM to human-like BFR subunit dimer (6 μM) in MES buffer (0.1 M, pH 6.5, 25 °C).

A key observation for human H-chain ferritin is the rapid rise and decay of a μ-1,2-peroxodiferric intermediate which absorbs at 650 nm (Bou-Abdallah et al. 2002). Although the 1,2-peroxodiferric intermediate has been suggested to occur in BFR, formation of intermediates with absorbances in the 550 nm region were not detected for this protein (Le Brun et al. 1993b). If the “human-like” BFR variant oxidizes iron by the same mechanism as does the human H-chain, the μ-1,2-peroxodiferric intermediate should be detectable. A multi-wavelength stopped-flow instrument was used to observe the spectroscopic changes after aerobic mixing of Fe(II) with the “human-like” BFR, and the characteristic change in absorbance at 650 nm was not observed as was the case for human H-chain ferritin (Figure 3.25, 1.7B).
Figure 3.25. Stopped-flow measurements of $\mu$-1,2-peroxodiferric intermediate in human-like BFR subunit dimer variant. Fe(II) was added to final concentrations of 432 $\mu$M to human-like BFR (108 $\mu$M) in MES buffer (0.1 M, pH 6.5, 25 °C) in a 2 cm path length cell. A rapid rise and decay of a species absorbing at 650 nm is not observed as reported for human H-chain ferritin.

3.8 Measuring the $\mu$-1,2-peroxodiferric intermediate in BFR 24-mer

The early events of iron oxidation in BFR are not well understood. A common strategy for finding intermediates in oxygen activated reactions is to measure changes in the visible spectrum after rapid mixing with a stopped-flow instrument (Bollinger and Krebs 2006). This method led to the discovery of an iron(III)-tyrosinate intermediate with an absorbance maximum at 550 nm in bullfrog H-chain ferritin (Waldo et al. 1993; Waldo and Theil 1993). The 550 nm region was measured in BFR and the formation of this intermediate was not observed (Le Brun et al. 1993b). Similar measurements with various ferritins led to the discovery of the $\mu$-1,2-peroxodiferric intermediate with an absorbance maximum at 650 nm in the reaction pathways of human H-chain ferritin, horse spleen ferritin, bullfrog M-chain ferritin, and various Dps proteins and an
absorbance maximum at 600 nm in the reaction pathway of *E. coli* bacterial ferritin A (Treffry *et al.* 1995; Zhao *et al.* 1997; Pereira *et al.* 1998; Zhao *et al.* 2005). The μ-1,2-peroxodiferric intermediate has never been reported for BFR even though it is expected to occur due to the similarity of the BFR dinuclear site with those found in many ferritins and other dinuclear iron enzymes.

To understand better the early events of iron oxidation by BFR, multi-wavelength stopped-flow spectroscopy was applied to BFR. Upon aerobic addition of Fe(II) to *E. coli* BFR, a broad absorption maximum arises at 650 nm (Figure 3.26). This observation is surprising because no change in this region of the spectrum had been reported in previous reports of similar experiments with this protein. This absorption maximum increases quickly and then decreases very slowly. The maximum absorbance of this spectroscopic intermediate is low and does not decay quickly as reported for human H-chain ferritin (Figure 1.7).

![Figure 3.26. Formation of transient intermediate species observed by multi-wavelength stopped-flow after aerobic addition of Fe(II) to BFR 24-mer.](image)

Multi-wavelength stopped-flow measurements of Fe(II) addition to BFR at 25 °C. Iron was added to final concentrations of 432 μM to wild-type BFR (9 μM) in MES buffer (0.1 M, pH 6.5) in a 2 cm path length cell. (A) Spectra recorded at 50 ms intervals from 0-500 ms show an increasing absorption maximum at 650 nm. (B) Absorbance change at 650 nm following aerobic addition of Fe(II).
CHAPTER IV – DISCUSSION

4.1 BFR subunit dimer as model for BFR 24-mer

4.1.1 Crystal structure of BFR subunit dimer

The three-dimensional structure of the Glu128Arg/Glu135Arg variant unambiguously confirms the dimeric structure of the BFR subunit dimer and establishes that the three-dimensional structures of the individual monomers are nearly identical to those of monomers of wild-type BFR (van Eerde et al. 2006) (RMSD = 1.43 Å over all 158 Cα atoms). Notable differences, however, can be observed in the position of the small C-terminal α-helix, which participates in formation of the inter-subunit 4-fold channels in the wild-type. The inter-subunit interactions stabilizing the 4-fold channels do not involve Glu128 or Glu135 but are disrupted in the assembly-deficient variant, which leads to a small re-positioning of the C-terminal helix relative to the four-helix bundle (Figure 4.1). Omitting 15 residues from the C-terminal α-helix and using only residues 1-143 resulted in an RMSD of 0.48 Å. The structural similarity of the subunit dimer variant and a subunit dimer within the wild-type 24-mer, disregarding inter-subunit channels, indicates that the main features of the subunit dimer structure do not require the higher-order oligomeric assembly of the wild-type protein.
Figure 4.1. Structural comparison of BFR 24-mer with BFR subunit dimer. The ribbon diagrams of (A) BFR wild-type (PDB ID: 2HTN) and (B) BFR subunit dimer are shown as viewed from the outer surface. The BFR subunit dimer is coloured by RMSD (blue-low, red-high). The largest deviation is seen in the small alpha helix at the C-terminal which is normally a part of the 4-fold symmetry channel.

4.1.1a Dinuclear iron site

Comparison of the subunit dimer and the wild-type 24-mer structures reveals interesting differences in and around the dinuclear iron site. For example, the first position of the dinuclear site of the subunit dimer variant is only partially occupied by zinc and the other half of the site is vacant. His130, which normally binds the metal ion in the second site, is found in an alternate, open conformation that presumably results from the vacancy at the second site. The RMSD calculated over all atoms in the dinuclear iron site between the subunit dimer and wild-type protein is 1.37 Å and 0.43 Å when calculated with and without the inclusion of His130, respectively. The His130 in the subunit dimer is expected to move from the “open” state to the “closed” state to bind metal ions once the dinuclear iron site becomes completely filled based on the structures of BFR with completely filled dinuclear iron sites. Complete iron binding at the dinuclear
iron site of the subunit dimer is indicated by normal Phase 2 ferroxidase activity which reaches saturation after addition of the full complement of iron atoms (Figure 3.6). In previous crystallographic studies of this species of BFR, occupancy of the dinuclear site has varied from full occupancy manganese, occupancy by a combination of iron and zinc, and full occupancy by zinc (Frolov et al. 1994; Dautant et al. 1998; van Eerde et al. 2006; Willies et al. 2009).

4.1.1b Ferroxidase pore

Cryoprotection of crystals with ethylene glycol serendipitously resulted in integration of one ethylene glycol molecule into a pocket that opens on the outer surface of the monomer and another ethylene glycol molecule that is integrated into a corresponding pocket that is accessed from the inner surface of the monomer and provides access to the dinuclear centre. The ethylene glycol molecule integrated into the outer surface of the protein is located between the ferroxidase site and the ferroxidase pore, slightly off centre to the side. It is in a position that corresponds to that of a so-called ferroxidase pore that has been described in the structure of *D. desulfuricans* (Macedo et al. 2003), and that was suggested to be the route by which iron enters the ferroxidase site and iron core of that protein. The observation of ethylene glycol in the ferroxidase pore provides evidence that small molecules can enter this pore to reach the dinuclear centre. Superposition of the BFR subunit dimer and BFR from *D. desulfuricans* shows the ethylene glycol to be at a position occupied by Tyr106 in *D. desulfuricans*, a residue lining the ferroxidase pore. In *E. coli* BFR, ethylene glycol is able to occupy this space because a wider pocket is available due to the smaller size of the corresponding
residue, Leu101. In the subunit dimer structure, residue Asn17 occupies two conformations as described in Section 3.1. The altered position of the ferroxidase pore that results from the altered conformation of Asn17 is unlikely to be important because it is not observed in any other structures and is likely to result simply from binding of the ethylene glycol molecules. Another example of a small molecule occupying a ferroxidase pore was recently discovered through crystallographic analysis of BFR from *Brucella melitensis* (PDB ID: 3FVB). This structure revealed imidazole bound to iron at the ferroxidase site such that the imidazole is located in the ferroxidase pore directly between the ferroxidase site and the pore opening.

### 4.1.2 Iron oxidation kinetics of BFR subunit dimer

Phase 2 of the ferroxidase reaction catalyzed by the BFR subunit dimer occurs in a manner similar to that of wild-type BFR. The rate of iron oxidation increases with increasing iron concentrations until the dinuclear iron site becomes saturated. At iron concentrations where the dinuclear iron site becomes completely occupied, a plateau is reached where additional iron is oxidized in Phase 3. Interestingly, at iron concentrations exceeding the amount required to completely fill the dinuclear iron site, the absorbance at 340 nm decreases slightly after completion of Phase 2. This pattern of iron oxidation has been observed previously for wild-type BFR when 1 mM of phosphate is added to the reaction mixture (Aitken-Rogers *et al.* 2004). In the present experiments, the buffer used for studies of the ferroxidase kinetics of the BFR subunit dimer should not contain any phosphate as the protein was repeatedly exchanged with MES buffer by centrifugal ultrafiltration. It is possible that the BFR subunit dimer behaves differently from the
wild-type and exhibits this pattern of oxidation even in the absence of phosphate. An interesting observation in the crystal structures of metal-free BFR grown with and without phosphate is the variation in conformation of His130 even without any clear phosphate interactions with the protein (Crow et al. 2009). The decrease in absorbance after completion of Phase 2 is also seen in Phase 3. A short lag phase is observed between Phase 2 and Phase 3 where the absorbance at 340 nm decreases briefly before increasing. The decrease in absorbance is only observed when iron is added in amounts that are in excess to that required to saturate the dinuclear iron site, which suggests that the change in absorbance is associated with the nucleation process. This process may involve rearrangement of ligands or electron transfer reactions that are not well understood.

4.2 Role of residues 47, 50, and 126

The early events of iron core formation in ferritins and ferritin-like proteins are believed to occur with the assistance of nucleation sites located at the inner surface of the spherical protein. The crystal structures of human L-chain ferritin, mouse L-chain ferritin, and DpsA from *Halobacterium salinarum* have allowed observation of iron or other metal ions bound to proposed nucleation sites (Levi et al. 1994; Harrison and Arosio 1996; Granier et al. 2003; Zeth et al. 2004; Wang et al. 2006). In these cases, the ligands for iron are glutamyl residues. Examination of the BFR crystal structure reveals a patch of acidic residues located within 10 Å of the dinuclear iron site on the inner surface of the protein that faces the iron core (Figure 4.2). The current results provide evidence that these residues are important for iron core nucleation through analysis of the ferroxidase
kinetics exhibited by variants in which these amino acid residues have been replaced with the corresponding amidated (neutral) residues.

**Figure 4.2. Electrostatic potential of BFR subunit dimer.** The “inner” surface of the BFR subunit dimer is shown and coloured by electrostatic potential (blue-positive, red-negative). The exposed heme is located between the two subunits and colored green. Dinuclear iron sites are located in each subunit and are indicated by dashed circles. Patches of negatively charged residues are located near the dinuclear iron sites. The proposed nucleation site residues are located in these areas. Figures generated from PDB ID: 2HTN.

### 4.2.1 Putative nucleation site residues

The ethylene glycol on the inner surface of the Glu128Arg/Glu135Arg variant is surrounded by the acidic residues Glu47, Asp50 and Asp126 that have been speculated previously as participating either in guiding of Fe(II) to the unoccupied dinuclear iron centre or nucleation of the iron core (Baaghil et al. 2003; Malone et al. 2004; Lewin et al. 2005) However, clear evidence that these residues are involved in such processes has not
been reported until now. To evaluate the potential functional role of these residues in the BFR subunit dimer, the ferroxidase kinetics of variants in which residues Glu47, Asp50 and Asp126 were replaced individually and in combination by the corresponding amides were investigated. Stopped-flow measurements established that the Glu47Gln and Glu47Gln/Asp50Asn/Asp126Asn variants possess markedly decreased Phase 2 iron oxidation activity relative to the wild-type protein and the Asp50Asn and Asp126Asn variants. These results clearly support a significant role for Glu47 in the ferroxidase activity of BFR. The mechanistic origin of this effect could result simply from the closer proximity of this residue to FE2 than is the case for Asp50 or Asp126 as noted above. As well, the alternate conformations of this residue in the partially bound and fully bound dinuclear iron sites may also be a factor. The Phase 3 activity of the Glu47Gln variant is also diminished but the effect is not attributable to a decrease in nucleation activity because this activity depends also on Phase 2 activity, which is also diminished. Both the Asp50Asn and Asp126Asn variants exhibit diminished Phase 3 reactivity. This result supports a role for Asp50 and Asp126 in the nucleation step of BFR iron core formation. Notably, these modifications of the electrostatic properties of the inner surface of the dimeric variant are sufficient to impede oxidation of Fe(II) in the absence of changes to the corresponding outer surface of the protein where the outer ferroxidase pore is located.

Two of the three residues that were substituted in this work, Asp50 and Asp126, are adjacent to residues forming the dinuclear iron site, Glu51 and Glu127, which are essential for catalytic activity. Therefore, the effect on iron oxidation observed for these variants could arise from perturbations to the ferroxidase site. However, major changes in the structure of the protein were not observed by UV-Vis or CD spectroscopy, indicating
that the heme binding environment and secondary structure of the protein of these variants are unchanged from those of the wild-type protein.

Along with the alternative conformations of Glu47 and His130 in the subunit dimer structure, His46 is also observed to have an alternative conformation. This residue is of interest because it is close to the three acidic residues and has been shown to also be important in iron core nucleation activity (Crow et al. 2009). Through the use of X-ray crystallography, site-directed mutagenesis and analysis of ferroxidase kinetics, His46 and Asp50 were shown to be important in iron core nucleation activity (Crow et al. 2009).

4.2.2 Conformational changes of Glu47/His130

Iron has been proposed previously to be translocated from the dinuclear iron site to the iron core by movements of residues His59 and Glu131 in D. desulfuricans BFR and residues Glu47 and His130 in A. vinelandii BFR (Liu et al. 2004; Swartz et al. 2006). The observation of a similar conformational change by residues Glu47 and His130 in the current structure suggests that a structural change of this type upon iron binding or release is characteristic of this family of proteins. Frolow and Kalb have reported that the uranyl derivative of E. coli BFR exhibits a similar movement of Glu47 and His130 for the binding of uranyl in the dinuclear iron site (Frolow and Kalb 2001). The crystal structure of B. melitensis BFR with only partial metal ion occupancy at the dinuclear iron site displays a similar arrangement for Glu47 and His130. The crystal structure of E. coli BFR with dinuclear sites fully occupied with a mixture of zinc and iron shows His130 to have a high degree of flexibility, indicating movement of this residue. In the present study, Glu47 is found to have altered conformations in the E. coli BFR structure.
containing zinc in the dinuclear site even without the accompanying altered conformation for His130. The dual conformations found for Glu47 and His130 suggest an involvement of these residues in a mechanism of iron binding and release which is yet unknown. With the dual conformations found for His130 being in an “open” or “closed” position providing a gate for the dinuclear site, a model involving translocation of the iron from the dinuclear site to the iron core after oxidation at the ferroxidase site is supported by the crystal structures of various BFRs, although it does not agree with previous iron oxidation kinetic studies. Specifically, iron is believed to occupy the dinuclear iron site after iron oxidation because the Phase 2 activity is not regenerated (Le Brun et al. 1993b; Yang et al. 2000). Regeneration of Phase 2 activity is expected if iron bound at the dinuclear site leaves this site after it is oxidized. The observation of mononuclear Fe(III) after iron oxidation by EPR measurements supports rearrangement of the dinuclear site after oxidation but further studies show this species to constitute a very low percentage of the sites (Le Brun et al. 1993a; Le Brun et al. 1993b; Yang et al. 2000).

Another residue of interest which is found to have more than one conformation in the subunit dimer structure and various wild-type structures is Tyr25. Tyr25 is located near the dinuclear iron site and can form hydrogen bonds to Glu47 and Glu94, a ligand for FE2 in the dinuclear site. Tyr25 is hydrogen bonded to Glu47 in E. coli BFR structures in which the dinuclear site is fully occupied by manganese or a mixture of iron and zinc, but this hydrogen bond is not indicated in the structure of E. coli BFR with the dinuclear site fully occupied by zinc or in the BFR subunit dimer structure with a dinuclear site partially occupied by zinc where the Glu47 is found in a different
conformation. The function of Tyr25 is not yet known, but this residue is thought to be essential for ferroxidase activity (Ma et al. 1999).

4.2.3 Subunit dimer QNN variant structure

Among this family of variants, the ferroxidase activity of the QNN variant is the most greatly diminished. To investigate the structural basis for this poor activity, the crystal structure of this variant was determined with protein that had been prepared in a manner that would deplete the sample of metal ions. As a result, no electron density attributable to bound metal ions was observed at the dinuclear active site of the variant. In addition, ethylene glycol molecules were not observed in this structure. The only difference between the procedures used for preparation of crystals of the QNN variant and for preparation of crystals of the subunit dimer variant is that the former was grown in a solution containing ethylene glycol and the latter was soaked in solutions containing ethylene glycol after crystal growth. The lack of bound ethylene glycol in the QNN variant is likely the result of the altered crystallization protocol. Dual conformations of Asn17 are seen in the subunit dimer variant but not in the QNN variant. This observation supports the hypothesis that the second conformation of Asn17 is caused by the binding of ethylene glycol.

After the crystallographic observation of metal ions occupying half of each dinuclear site in the structure of the subunit dimer variant, the demetallation procedure was changed to remove all metal ions more stringently. As expected, metal ions are absent from the dinuclear iron site of the QNN variant structure. In a completely empty state, the His130 of the ferroxidase site is in the “open” position as suggested to be the
resting state before iron binding. All structures obtained thus far of the subunit dimer and variants of are found with the His130 in the “open” position. Although His130 is expected to be able to move to the “closed” position because Phase 2 activity is observed for the subunit dimer variant for up to two iron ions per ferroxidase site, this position of H130 has not been observed crystallographically.

Attempts to obtain a crystal structure of the subunit dimer with a fully occupied dinuclear iron site were unsuccessful. Crystals, grown from protein to which sufficient levels of iron had been added to saturate the ferroxidase site, contained only partially occupied dinuclear sites (results not shown). The iron atoms presumably exited the dinuclear iron site during crystal growth. Alternative approaches that might produce crystals with iron occupying both halves of the dinuclear site might involve soaking the crystal in iron or growing crystals with protein containing zinc saturated dinuclear sites inasmuch as zinc binds to the dinuclear site with greater affinity than does iron.

The most interesting feature of the QNN variant structure is the patch of negatively charged residues which are replaced with the corresponding amides. In the subunit dimer variant, these residues form an opening to the dinuclear iron site when His130 is in the “open” position. In the QNN variant, removal of the negative charges from the three residues resulted in closure of this possible route for iron to the dinuclear site. The collapse of the opening is probably the result of the lack of repulsion between the normally charged amino acids. New hydrogen bonds are formed by Gln47 with Asn50, Gln51, and Asn126. Thus these amino acid substitutions not only removed the negative charge that might provide an electrostatic gradient for attracting iron ions to the dinuclear
site, they also resulted in blockage of a possible route of iron entry to the dinuclear iron site.

The other possible route from the solvent to the dinuclear site is through the ferroxidase pore located on the “outer” surface. No changes in the ferroxidase pore of the QNN variant are observed which would impair the movement of iron through this route. The ferroxidase site in the QNN variant appears to be catalytically functional based on its structural similarity with the ferroxidase site in the subunit dimer. Therefore, the diminished ferroxidase activity exhibited by the QNN variant may result from impeded entry of iron to the dinuclear iron site through the opening at the “inner” surface. Iron entry to the ferroxidase site through the inner surface would imply that iron must first enter the core of the 24-mer protein through one of the channels described that provide access through the protein shell. Another possible explanation for the lower ferroxidase activity of this variant is the inability of Gln47 to adopt the two conformations previously observed as the result of the new hydrogen bonds formed by this residue in the QNN variant. Moreover, in the wild-type protein, Glu47 forms a hydrogen bond with Tyr25 and this interaction may be important for the normal functioning of the dinuclear site.

4.2.4 Nucleation sites in BFR 24-mer

Site-directed mutagenesis and iron oxidation kinetics were used to determine whether the nucleation site residues identified in the subunit dimer variant have a similar function in the wild-type 24-mer. The ferroxidase kinetics of the variants in the subunit dimer showed that the mutations Glu47Gln or Glu47Gln/Asp50Asn/Asp126Asn substitutions resulted in greatly diminished Phase 2 reactivity. Introduction of these same
substitutions into the 24-mer also led to a protein with compromised ferroxidase activity, but the diminution in activity was not as great. The 24-mer retains more activity than the subunit dimer when these residues are substituted, which suggests that the oligomerization of the subunit dimers contributes to maintenance of function. This result is also observed in comparing the activities of the subunit dimer variant and the 24-mer in which the ligands at the dinuclear site are replaced with residues that occur at the dinuclear site of the human ferritin H-chain. Once again, the effect of these substitutions on activity is greater in the case of the subunit dimer variant than for the 24-mer. The decreased stability of the subunit dimer variant relative to that of the 24-mer might make the smaller protein more susceptible to indirect structural perturbations resulting from these amino acid substitutions. Although the subunit dimer variant retains ferroxidase activity, its structure is slightly altered compared to that of the 24-mer as noted above (p.97). Introduction of additional amino acid substitutions to the subunit dimer may synergistically cause structural deviations that result in decreased ferroxidase activity relative to the 24-mer.

The surface of the subunit dimer variant that normally faces the iron core in the 24-mer is exposed to the solvent, so as the iron core assembles on the surface of this smaller protein, the solubility of the protein decreases (Malone et al. 2004). As a result, the subunit dimer variant precipitates after binding a relatively small amount of iron relative to the amount that the 24-mer can bind without precipitation. The much greater iron binding capacity of the 24-mer allowed addition of greater amounts of iron to the protein for the analysis of Phase 3 kinetics, which increased the contribution of Phase 3 relative to Phase 2 to the change in absorbance at 340 nm. Thus, the effect of the
substitutions at the nucleation site on the Phase 3 kinetics is more distinct for the results obtained with the 24-mer. The amount of iron added to the subunit dimer during evaluation of Phase 3 is sufficient to fill the dinuclear iron site and have one iron remaining to bind the nucleation site in each subunit. In the 24-mer variants, the amount of iron added is sufficient to fill the dinuclear iron site and have 352 irons remaining per 24-mer to build the core. In the latter experiments, a small cluster of iron is expected to bind to the nucleation sites of the 24-mer and serve as the core formation surface for additional irons. A select number of iron clusters are postulated to dominate the core formation process. It is interesting that for the variants in which putative nucleation sites have been substituted, the rate of core formation is low throughout the entire process even after the nucleation event should have already occurred. However, at least for the Asp126Asn variant, the rate of iron oxidation increases with time. Iron oxidation still occurs at an appreciable rate in the variants where only a single residue is altered, presumably because the other residues are still able to function albeit with reduced efficiency. According to the linked transfer model, electrons are shuttled between the ferroxidase site and the iron core. The nucleation site residues may be required to bind iron properly within close proximity to the ferroxidase site for this process to work optimally.

4.2.5 Bacterioferritin sequence alignment

To evaluate the possibility that the acidic residues studied in the current work might participate similarly in the ferroxidase activity of other bacterioferritins, the sequences of several members of this protein family are aligned in Figure 4.3. From this analysis, it is
clear that each of these residues is highly but not absolutely conserved, so it seems highly likely that the functional contributions of these residues observed in the present study would also be observed for these other species of the protein. In those unusual cases where one of these acidic residues is not conserved, an adjacent acidic residue may occupy the same stereochemical position, but confirmation of this possibility must await detailed characterization of these other proteins. Tyr25 is present in all the BFRs included in this sequence alignment.

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<tr>
<th>Bacterial Species</th>
<th>Sequence Alignment</th>
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<tr>
<td>D.desulfuricansG20</td>
<td>MTASGEERANVLEVKVARMELIAHMYQHNYGHG 37</td>
</tr>
<tr>
<td>D.desulfuricansATCC27774</td>
<td>MAGHDEERANVLEVKVARMELIAHMYQHNYGHG 36</td>
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<tr>
<td>B.melitensis</td>
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<tr>
<td>R.leguminosarum</td>
<td>MKGDKVIEFLAELFELGAVNQYVHHR 31</td>
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<tr>
<td>R.capsulatus</td>
<td>MKGDKVIEFLAELFELGAVNQYVHHR 31</td>
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<td>P.aeruginosaA</td>
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<tr>
<td>P.aeruginosaB</td>
<td>MKGDKVIQNLKILGELIAINQYFHIHSMR 31</td>
</tr>
<tr>
<td>SynechocystisA</td>
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<tr>
<td>SynechocystisB</td>
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<tr>
<td>B.cereus</td>
<td>LDMDYGLNQDVYETSHEDVHYVTILFGPRLNQVLKLAB 81</td>
</tr>
</tbody>
</table>
Figure 4.3. Alignment of amino acid sequences of several bacterioferritins. The sequences were aligned with the Clustal W2 program (Larkin et al. 2007). The acidic residues replaced in the current work are indicated by arrows. The ferroxidase centre residues are indicated with "+". The "*" indicates that the residues are identical in all sequences in the alignment, "::" indicates conserved substitutions, and "." indicates semi-conserved substitutions.

4.2.6 Nucleation sites in BFR and mammalian ferritins

The primary and three-dimensional structures of human H-chain and L-chain ferritin and E. coli BFR were aligned to determine the similarities and differences between the nucleation sites (Figure 4.4). Glu47, which has a special role in BFR, is not conserved in human ferritin. However, both H-chain and L-chain ferritins have glutamates at the positions corresponding to Asp50 and Asp126 of BFR.
Figure 4.4. Alignment of amino acid sequences of BFR and HuFTN. The sequences are aligned by the Clustal W2 program. Glu47, Asp50, and Asp126 of BFR are indicated by arrows. The "**" identifies residues that are identical in all aligned sequences, ":." indicates conserved substitutions, and ":." indicates semi-conserved substitutions.

The nucleation site residues in H-chain ferritin were believed to be Glu61, Glu64, and Glu67 (Lawson et al. 1991; Wade et al. 1991; Levi et al. 1992; Levi et al. 1994; Crichton et al. 1996). Substitution of these residues in combination (Glu61Ala/Glu64Ala/Glu67Ala) resulted in diminished ferroxidase activity. More recent studies of the Glu64Ala/Glu57Ala variant indicate, however, that Glu64 and Glu67 are not important in core formation as previously thought because this variant exhibits normal ferroxidase activity and mineralization (Bou-Abdallah et al. 2004). In the structure of the wild-type H-chain, Glu61, which corresponds to Asp50 in BFR, occurs in two conformations, one of which places it in position to participate in formation of the ferroxidase site. In part for this reason, the decreased activity of the Glu61Ala/Glu64Ala/Glu67Ala variant can be attributed to disruption of the ferroxidase site rather than the nucleation sites. Glu140 in human H-chain ferritin occupies the
position corresponding to Asp126 in BFR, and its role in iron core formation has not been investigated.

Glu57, Glu60, Glu61, and Glu64 (H-chain numbering) are believed to be nucleation site residues in the L-chain ferritin (Wade et al. 1991; Santambrogio et al. 1996; Granier et al. 2001; Wang et al. 2006). Glu57, Glu60, Glu64, and Glu67 of L-chain ferritin occupy the positions equivalent to His46, Ile49, Asp50, and Lys53 in BFR, respectively. In BFR, His46 and Asp50 are believed to be involved in iron core nucleation, but Ile49 and Lys53 appear not to be involved. Similarly to the H-chain ferritin, Glu140 in L-chain ferritin occupies the corresponding position of Asp126 in BFR and is believed to play a role in iron core formation. Glu140 is conserved in mammalian ferritins except those from mouse and rat, which have a lysine at this position (Santambrogio et al. 2000). This lysine is significant because it interacts with Glu61 and thereby decreases efficiency of iron incorporation (Granier et al. 2001).

4.3 Channels in BFR

4.3.1 Subunit dimer variant as a tool for evaluating putative functions of channels

Zinc, a well known inhibitor of BFR ferroxidase activity, is thought to act by binding to the dinuclear iron sites or the inter-subunit channels, and blocking access of iron to these sites (Le Brun et al. 1996; Baaghil et al. 2003; Bou-Abdallah et al. 2003). One of the advantages (or disadvantages) of utilizing the subunit dimer for kinetic studies is the lack of inter-subunit channels. By measuring the effects of zinc on ferroxidase kinetics of the subunit dimer, any reduction in activity is not attributable to binding and blockage of the inter-subunit channels. The observation that zinc inhibits iron oxidation
in the subunit dimer provides evidence that inhibition of iron oxidation occurs through binding of zinc at the ferroxidase site. Nevertheless, while the decreased efficiency of iron oxidation caused by zinc proves the inter-subunit channels are not required for inhibition, it does not prove that zinc does not bind to the inter-subunit channels when they are present. Structural evidence favors the mechanism of inhibition by zinc binding at the dinuclear iron sites. Dinuclear iron sites completely filled with zinc or with mixtures of iron and zinc have been observed in crystal structures of the wild-type *E. coli* BFR (van Eerde *et al.* 2006; Willies *et al.* 2009). Partial occupancy of the dinuclear iron site with zinc is also observed in the subunit dimer structure determined in the current work. In all of these structures, zinc is observed only at the dinuclear iron sites and nowhere else in the structures.

The total amount of iron oxidized does not decrease proportionally to the amount of zinc added. A dramatic reduction (~46%) in the total amount of iron oxidized is observed when a 1:1 equivalent of zinc is added to BFR subunit dimer (2 dinuclear iron sites, 4 binding sites) (Figure 3.23C). If all the zinc is binding the dinuclear iron sites in a pairwise fashion, then the greatest decrease in the amount of iron oxidized should be just 25% with this amount of zinc. This greater decrease in iron oxidation can be explained if only half of each dinuclear site needs to be blocked by zinc before it is incapacitated. Mutagenesis of key ferroxidase residues shows that the dinuclear iron site requires both halves of the site to be functional because iron is oxidized in a pairwise fashion (Baaghil *et al.* 2003). Therefore, addition of 1 equiv of zinc for each dinuclear centre to the BFR subunit dimer variant can result in a maximum decrease of 50% in the amount of iron oxidized if all the added zinc binds to just one position within each dinuclear site.
Mixtures of partially and fully occupied dinuclear sites are expected to create the observed levels of total iron oxidized at various zinc concentrations. The distribution of zinc binding to the two halves of the dinuclear centre as zinc is added to the subunit dimer variant initially devoid of metal ions is complicated by inequivalent binding affinities of the two halves of the dinuclear sites and possible cooperative binding effects within the dinuclear sites and between the subunits.

4.3.2 Iron entry channels in BFR

One of the differences between the subunit dimer and the 24-mer is the presence of inter-subunit channels in the latter protein. Therefore, studies to explore which channels are used for iron entry require the use of the 24-mer. If the channels used for iron entry in the 24-mer can be disrupted by site-directed mutagenesis of residues that interact with iron, then the effect can be reversed in the subunit dimer which does not have the restrictive flow of iron through channels as the “inner” surface is already exposed to the solvent. Nevertheless, the ferroxidase pore is retained in the subunit dimer because it is defined solely by the amino acid residues of a single subunit.

The channel used by iron for entering the central cavity is expected to be hydrophilic. Negatively charged residues are expected to assist with the attraction of iron ions to the channel. Mutation of the negatively charged residues in human ferritin results in diminished iron binding and decreased ferroxidase activity (Treffry et al. 1993; Levi et al. 1996; Bou-Abdallah et al. 2008). Examination of the structure of E. coli BFR reveals the 3-fold, 4-fold, and B-site channels and the ferroxidase pore as possible routes for iron to reach the inner core through the proteinaceous shell.
Asp109, Asn148, Asp139, and Asp96 were replaced to disrupt the 3-fold, 4-fold, and B-site channels and the ferroxidase pore, respectively. In selecting residues to substitute at these positions, preference was given to replacing negatively charged residues normally found at the entrances to the channels and pore. Aside from the 4-fold channel, an aspartate is found near the entrance of all the channels and the pore. An asparagine was chosen for replacement at the entrance to the 4-fold channel because no charged residues occur at that site. In three of these substitutions, phenylalanine was introduced to maximize the chance of disrupting the normal function of the channels by not only removing the charge but also possibly blocking the entrance of the channel. The exception to this strategy was Asp96 of the ferroxidase pore which was replaced with asparagine as was the case for the nucleation site variants.

Notably, replacement of a single residue in each monomer to modify the 3-fold and 4-fold channels has an amplified structural effect that results from the symmetrical arrangement of the subunits to form the channel. Specifically, replacement of one of these residues in a subunit results in alteration of three of the residues in the 3-fold channel and four of the residues in the 4-fold channel. However, the ferroxidase pore and the B-site channel are not formed by a similar symmetrical arrangement of monomers, so in these cases the structural consequences of replacing a single residue on the structure of the pore or channel is not as great. For example, the neutralization of one charged residue in the B-site channel, which is normally lined with five negatively charged residues, could result in a relatively small structural perturbation that could make the functional consequence of the substitution less easily detected.
Despite these considerations, the ferroxidase kinetics of these variants did not help distinguish which of the channels are used by iron for entering the core (Figure 3.21, 3.22, p.89-90). This result would be explained if iron enters the cavity through both the 3- and 4-fold channels. However, the fact that the Asp109Phe/Asn148Phe double variant exhibited ferroxidase activity similar to that of the wild-type protein and each of the individual variants is inconsistent with this conclusion. At present, therefore, it appears that either these substitutions were insufficient to disrupt the function of the channels and pore significantly or that other pathways not evident from the crystal structure are used for iron entry.

Interestingly, the ferroxidase activity of the Asp96Asn variant exhibited the greatest increase in activity relative to the wild-type. The ferroxidase pore is composed mainly of hydrophobic residues leading to the ferroxidase site, which would be favourable for the entry of similarly hydrophobic reactants. The observation of ethylene glycol in the crystal structure of the subunit dimer shows the ability of small molecules to enter the pocket leading from the outer surface to the ferroxidase site. A possible role for the ferroxidase pore is the access of dioxygen to the active site. This role would explain the increase in ferroxidase activity exhibited by this variant because removal of the negative charge could favour the transfer of oxygen to the ferroxidase site. The homologous pore in human ferritin is also hydrophobic and has been postulated to serve as a dioxygen entry pathway based on molecular dynamic simulations (Ciacchi and Payne 2004).

Asp109 and Asn148 at the 3-fold and 4-fold symmetry channels were also replaced with a lysyl residue with the expectation that the resulting positive charge would
repel the iron. Unfortunately, these variants could not be evaluated owing to their low solubility. The aggregation exhibited by these variants is most likely attributable to electrostatic interactions between BFR molecules created by the presence of these new positive charges.

4.4 Reaction mechanism at the ferroxidase site

4.4.1 “Human-like” ferroxidase site in BFR

The mechanism by which human H-chain ferritin oxidizes iron involves the cyclical regeneration of the dinuclear iron site (Sun et al. 1993; Yang et al. 1998). On the other hand, iron oxidized at the dinuclear iron site of BFR is believed to remain bound at the dinuclear site to form a *de facto* co-factor and does not migrate from this site to the growing iron core as observed for human H-chain ferritin (Le Brun et al. 1993b; Yang et al. 2000). This difference in mechanisms is believed to result from subtle structural differences between the ferroxidase sites of these proteins. Inspection of the dinuclear iron sites of human H-chain ferritin, *E. coli* ferritin A, and bullfrog ferritin reveals the commonality of asymmetrical sites that include a glutamyl residue that bridges the two iron atoms and one histidyl ligand that binds to Fe A and not Fe B (Figure 1.2). Conversely, enzymes that use the dinuclear iron site as a cofactor (e.g., stearoyl-acyl carrier protein Δ9-desaturase, ribonucleotide reductase, and methane monooxygenase) have more symmetrical sites in which the iron atoms are bridged by two glutamyl residues and two histidyl ligands are bound to Fe A and Fe B. The dinuclear iron site of BFR appears to resemble these dinuclear iron enzymes than it does human ferritin H-chain (Figure 1.2, p.12). BFR has a symmetrical dinuclear site with two glutamyl
residues that bridges the two iron atoms and two histidyl residues that bind to Fe A and Fe B.

The protein ligands provided to the dinuclear iron sites of human H-chain ferritin and E. coli BFR differ in the identity of two residues. However, structural alignment demonstrates that three residues at the dinuclear site of BFR must be replaced to convert this site to recreate a dinuclear iron site similar to that found in human H-chain ferritin. Glu61 of human H-chain occurs in two conformations that allow it to participate in the dinuclear iron site as well as the nucleation sites. The Asp50 of BFR, which occupies the position equivalent to Glu61 in human H-chain ferritin, would be required to exhibit similar conformational flexibility for it to participate in iron binding at the dinuclear site. Replacement of single residues at the dinuclear iron site (Glu18Ala, Glu127Gln, His130Glu) have been shown to disrupt the iron core formation process (Baaghil et al. 2003). However, the Asp50Glu/Glu127Gln/His130Ala substitutions in the 24-mer protein create what appears to be a variant with human H-chain ferritin-like activity (Moore et al, unpublished results), and the ferroxidase reaction kinetics exhibited by this variant are not altered greatly from those of the wild-type protein. Nevertheless, the ferroxidase kinetics of these two proteins are not identical. Following addition of Fe(II) to the variant, the absorbance at 340 nm increases gradually, and no Phase 2 associated burst is observed. Surprisingly, the Asp50Glu/Glu127Gln/His130Ala substitutions in the subunit dimer studied in the current work result in greatly diminished ferroxidase activity. Presumably, the dinuclear iron sites of the subunit dimer are sufficiently disrupted by these substitutions to cause a decrease in its ferroxidase activity. The reasons for differing effects of these identical substitutions in the 24-mer and the subunit dimer are unclear at
present, but the greater instability of the subunit dimer relative to the 24-mer may be a factor (Kilic et al. 2003). Another factor may be the greater importance of an existing core in the human-like variants for iron oxidation. Because the 24-mer has a much greater iron binding capacity than does the subunit dimer variant, more iron can be added to it (without the risk of precipitation) which allows faster core formation than is possible for the subunit dimer. Addition of a great excess of iron to the latter protein results in insolubility induced by formation of a small iron core because in this case, the iron core is exposed to bulk solvent.

If the mechanism of the ferroxidase reaction catalyzed by the human-like BFR variant is the same as that employed by human H-chain ferritin, then formation and decay of a $\mu$-1,2-peroxodiferric intermediate should be detected by rapid mixing kinetics. However, the $\mu$-1,2-peroxodiferric intermediate was not observed for the Asp50Glu/Glu127Gln/His130Ala subunit dimer variant in the current study. Even if this intermediate does form during the turnover of this protein, it might be difficult to observe because iron oxidation is slow. At the time of this work, rapid scanning stopped-flow kinetics analysis of the Asp50Glu/Glu127Gln/His130Ala 24-mer variant, which exhibits ferroxidase activity similar to that of wild-type BFR, had not been reported.

**4.4.2 Search for $\mu$-1,2-peroxodiferric intermediate**

Multi-wavelength stopped-flow spectroscopy was performed for BFR to determine the spectroscopic changes that occur as Fe(II) is added aerobically to BFR devoid of non-heme metal ions. Unexpectedly, a small absorbance maximum was observed at 650 nm (Figure 3.26, p.96). The observation of such a spectroscopic
intermediate is surprising because BFR is commonly cited as not exhibiting a detectable \( \mu \)-1,2-peroxodiferric intermediate. It is possible that this species was not observed previously due to difficulties in detection. The detection of this species in the current work may have been assisted by using a higher concentration of BFR than was the case in previous work. In addition, a cuvette with a 2 cm path length was used in this work rather than the standard 1 cm cuvette which essentially doubles the sensitivity of detection. Finally, previous studies (Le Brun et al. 1993b) anticipated the appearance of a maximum at 550 nm because those experiments were completed shortly after discovery of iron(III)-tyrosinate coordination in bullfrog H-chain ferritin, which had an absorbance peak at this wavelength. As it is now apparent that this corresponding absorbance maximum occurs at 650 nm, the previous experiments would have been handicapped by considering the wrong spectroscopic region. Subsequent studies with other ferritins lacking the iron(III)-tyrosinate interaction consistently observed formation of a \( \mu \)-1,2-peroxodiferric intermediate with absorbance maximum at 650 nm.

The \( \mu \)-1,2-peroxodiferric intermediate is also exhibited by a variety of diiron enzymes in addition to ferritin, e.g., methane monooxygenase hydroxylase, a variant R2 protein of ribonucleotide reductase, and stearoyl-acyl carrier protein \( \Delta 9 \)-desaturase (Broadwater et al. 1998; Moenne-Loccoz et al. 1998; Broadwater et al. 1999; Valentine et al. 1999). The similarity of the dinuclear iron sites in these proteins with the dinuclear site of BFR, therefore, led to the expectation that the \( \mu \)-1,2-peroxodiferric intermediate should also occur during the catalytic cycle of BFR. Consequently, the failure of previous experiments to detect this intermediate was attributed to formation and decay of the intermediate within the dead time of stopped flow mixing (~3-5 ms). Nevertheless, the
identity of the species detected in the current study is not completely unambiguous. A \( \mu \)-1,2-peroxodiferric intermediate in BFR is expected to decay with production of hydrogen peroxide and a \( \mu \)-1,2-oxodiferric species (Yang et al. 2000). Although the absorbance maximum at 650 nm is consistent with formation of a \( \mu \)-1,2-peroxodiferric intermediate as observed for other ferritins, the slow decay of this species is uncharacteristic of such an intermediate (Figure 3.26). Nevertheless, a stable \( \mu \)-1,2-peroxodiferric intermediate (\( t_{1/2} \sim 26 \) min) is observed in the case of stearoyl-acyl carrier protein \( \Delta \)9-desaturase (Broadwater et al. 1998). It is possible that this species does not form with every turnover of the ferroxidase active site and, so represents only a small portion of the BFR reaction cycles.

The inter-atomic distance between the two iron atoms in the \( \mu \)-1,2-peroxodiferric intermediate at the dinuclear iron site has been identified as a key factor in determination of the reaction mechanism of dinuclear iron proteins (Hwang et al. 2000). The relatively short iron-iron distance observed in ferritin promotes breakdown of the intermediate due a strained Fe-O-O angle that favours the production of hydrogen peroxide and \( \mu \)-oxo and \( \mu \)-hydroxo diferric species. Alternatively, formation of species with high valent iron oxidation states are found at the dinuclear sites of ribonucleotide reductase and methane monooxygenase (Lee et al. 1993; Bollinger et al. 1998; Broadwater et al. 1998; Mitic et al. 2007). Interestingly, the dinuclear iron site of BFR resembles that of ribonucleotide reductase and methane monooxygenase more than it does that of the ferritin H-chain, suggesting that formation of a \( \mu \)-1,2-peroxodiferric intermediate would result in formation of high valent iron species similar to those proposed for these enzymes. However, the reaction pathway for oxygen activation at the dinuclear iron site of BFR is
believed to follow the pathway observed for ferritins with the production of hydrogen peroxide and μ-oxo and μ-hydroxo diferric species rather than high valent iron species (Yang et al. 2000; Bou-Abdallah et al. 2002).

4.5 Future studies

Measurement of Phase 2 and Phase 3 iron oxidation kinetics of the nucleation site variants established that the Asp50Asn and Asp126Asn variants have similar Phase 2 activity but reduced Phase 3 activity relative to the wild-type protein. A substantial decrease in the Phase 2 rates were observed for the Glu47Gln and QNN variants. It would also be interesting to determine the rate of iron binding at the ferroxidase sites (Phase 1) of these variants. Phase 1 is normally much faster than Phase 2 or 3 and can be measured by observing perturbations of the heme Soret band by stopped-flow spectroscopy. Decreased rates of binding would support the hypothesis that these negative residues are important for attracting iron to the dinuclear iron site. As seen in the crystal structure of the subunit dimer QNN variant, the simultaneous alteration of the negatively charged residues results in blockage of the opening at the inner surface which was found to be filled with an ethylene glycol in the subunit dimer structure. The ferroxidase pore is unaffected in this structure, but it is not known if it can provide a route of iron entry to the ferroxidase site. Phase 1 kinetics of the subunit dimer QNN variant would be of particular interest because these measurements could help determine if iron is capable of entering the ferroxidase site through the ferroxidase pore or if the blockage at the inner pore prevents iron binding at the ferroxidase site. Such studies would also help determine if the decreased Phase 2 and 3 iron oxidation activity observed
for this variant is caused by a diminished rate of Phase 1. An advantage of using the subunit dimer variants for Phase 1 measurements is the relatively straightforward method required for reconstitution of the protein with heme to assure that all heme binding sites are occupied so that maximal sensitivity could be achieved in monitoring Phase 1.

The majority of kinetic studies concerning the BFR ferroxidase reaction have been performed with the protein from *E. coli*. Studies of BFR from other species are limited, and it would be valuable to evaluate the activity of BFR from other species for comparison. *R. capsulatus* BFR presents a particularly attractive target for such studies as its crystal structure is available (Cobessi *et al.* 2002) and the subunit dimer variant of *R. capsulatus* has been reported (Kilic *et al.* 2003). Based on the amino acid sequence alignment of BFRs from various species, the nucleation sites proposed in this dissertation are conserved in the BFRs from most bacteria. It would be interesting to test if the effects of altering these residues in *R. capsulatus* BFR would produce effects similar to those observed for *E. coli* BFR.

Phase 2 and 3 iron oxidation kinetic measurements of the channel variants did not help distinguish which of the inter-subunit channels are important for iron entry. However, even if the rate of iron entry is decreased in any of these variants, ferroxidase kinetics measurements may not be able to detect this involvement because it may not be the limiting step in iron oxidation. If iron predominantly uses the opening at the inner surface instead of the ferroxidase pore for entering the ferroxidase site, then iron is expected to first pass through one of the many inter-subunit channels before being able to access the inner pore. Therefore, obstructions at the inter-subunit channels should result
in decreased rates of iron binding at the ferroxidase sites. Phase 1 iron binding measurements would be of interest for the channel variants because this phase would presumably be more sensitive to small changes in the rate of iron entry than Phases 2 or 3.

Zinc is a well known inhibitor of BFR in vitro and is even capable of inhibiting BFR already containing an iron core. It would be interesting to determine if zinc is capable of inhibiting BFR in vivo. E. coli with inactivated fur and bfr genes were found to have greater sensitivity to hydroperoxides (Abdul-Tehrani et al. 1999). The inhibition of BFR in vivo can be tested by the sensitivity of fur mutants to hydroperoxides in the presence of zinc. The dinuclear iron sites of BFR and other diiron enzymes such as ribonucleotide reductase are similar, which suggests that they have similar metal ion binding properties. Indeed, the inhibition of ribonucleotide reductase by zinc has been observed (Oblender and Carpentieri 1990). The inhibition of ribonucleotide reductase is of interest because ribonucleotide reductase is required for cell growth and is a target for cancer treatment (Cerqueira et al. 2005). Based on the studies with BFR, inhibition of ribonucleotide reductase is most likely caused by binding of zinc at the dinuclear iron site. In addition to zinc, cadmium has recently been found to be a strong inhibitor of BFR (results not shown), and it would be interesting to test if cadmium inhibits ribonucleotide reductase similarly.

An unknown species that absorbs at 650 nm is observed to form in BFR. Although the wavelength of maximum absorbance corresponds to that expected for a μ-1,2-peroxodiferric intermediate found in many diiron enzymes, its formation and decay kinetics differ from those observed for such intermediates in other ferritins. The assignment of the μ-1,2-peroxodiferric intermediate in ferritin has been based on
Mössbauer spectroscopy, resonance Raman spectroscopy and EXAFS measurements (Moenne-Loccoz et al. 1998; Pereira et al. 1998; Hwang et al. 2000; Bou-Abdallah et al. 2002). The transient nature of this species required preparation of these spectroscopic samples by freeze-quench after addition of iron to trap the $\mu$-1,2-peroxodiferric intermediate. Variant forms of ribonucleotide reductase were used to stabilize and assist in the detection of the $\mu$-1,2-peroxodiferric intermediate (Bollinger et al. 1991; Bollinger et al. 1998; Moenne-Loccoz et al. 1998). Similar experiments with BFR would help assign the identity of the species observed in the current work and would provide insight into the mechanism of iron oxidation at the ferroxidase site.
REFERENCES


Supplementary figures

**Figure A1. Schematic diagram of PCR cloning strategy.** The PCR in step 1 produces the megaprimer. The megaprimer is used in the PCR of step 2 to integrate the BFR gene into modified pET 32b.
Figure A2. SDS-PAGE of representative purified protein samples. Coomassie Brilliant Blue-stained 15% polyacrylamide gels loaded with protein samples. A) Purified BFR subunit dimer from pALN18. Fractions 6-15 were pooled from this preparation. B) Purified BFR subunit dimer from pET32b-BFR. C) Purified BFR 24-mer from pALN1. Fractions 6-13 were pooled from this preparation.
List of publications

Parts of this dissertation have been published.