Why is there no carbonic anhydrase activity available to fish plasma?

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Abstract

Carbonic anhydrase (CA) is absent in the plasma of vertebrates. In vitro, CA in fish plasma will short-circuit the effect of catecholamines on the increase in red blood cell (RBC) pH and volume, both of which increase the hemoglobin affinity for O₂. CA was infused into trout for a period of 6h during which the animal was submitted to deep hypoxia (P_{O₂}= 30-35 torr) and during recovery after exhaustive exercise. During hypoxia, O₂ content, lactate, catecholamines, hematocrit, hemoglobin and pH_i were similar to the saline infused control group. On the other hand, cell volume was significantly higher and pH_e, total CO₂ and organic phosphates were significantly lower than the control group. The concentration of CA was not high enough to completely short-circuit the increase in pH_i and RBC volume caused by catecholamines. The lower pH in the CA infused animals could enhance the activity of the Na⁺/H⁺ pump which would keep the NTP low. CA in plasma, during hypoxia, did not cause the expected reduction in blood oxygen content but did have a marked effect on plasma total CO₂. During the recovery period of exhaustive exercise, lactate, catecholamines, hematocrit, hemoglobin, MCHC, P_{O₂}, HbO₂ and pH_i were similar to the saline infused animals. Total CO₂ and pH_e were significantly higher in the CA infused fish than in the saline infused ones. CA infusion, in this case, probably caused acid retention in the muscle. Acid efflux from the muscle would decrease pH_e or if the acid was excreted at the gills, bicarbonate would be titrated and the stores would be lower than in control animals. CA activity available to plasma would mean greater fluctuation of plasma pH, at least in hypoxic conditions, and red blood cell pH in general. pH is a

balance between acid loading at the muscle and acid excretion at the gills or the kidneys, we cannot distinguish between a decrease and an increase of one of the two which resulted in a decrease of plasma pH. Fish have a large Haldane effect and HCO_3^- flux through the red cell ensures that the protons are excreted as CO_2 and cannot bind again to hemoglobin. The absence of CA in the plasma ensures that HCO_3^- flux through the red cell is maintained.

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Chapter 1

General Introduction

Carbonic anhydrase: general information

Carbon dioxide is the major end product of aerobic metabolism. When CO₂ is transferred to the blood, most of it is hydrated to HCO₃⁻ according to the following equation:

Because the ionization of H₂CO₃ is very rapid, H₂CO₃ is often omitted in this reaction. From this reaction, it is evident that when CO₂ is added to a solution, pH decreases. Conversely, the addition of protons will affect CO₂ concentrations. It is not surprising, therefore, that the dynamics of this reaction are an important component of acid-base balance.

The enzyme carbonic anhydrase (CA) catalyzes the reversible CO₂ hydration/dehydration reaction. In the absence of CA the CO₂ equilibrium reaction is very slow, about 30 seconds at 10° C (Nikinmaa, 1992). This enzyme has one of the highest known turnover numbers ($k_{\text{cat}} = 10^4$ - 10^6 sec^{-1}) and is inhibited by a wide range of inhibitors with acetazolamide as the most commonly used (Dodgson, 1992).

There are 7 isoenzymes known in mammals (Dodgson, 1992). Two cytosolic isoenzymes are present in the red blood cells (CA I and CA II). CA II is also present in many other tissues like kidneys and lungs. By definition, the CA present in skeletal muscle is CA III and the one membrane-bound is CA IV. CA V is the mitochondrial isoenzyme, CA VI is secreted in the saliva and CA VII is found in the salivary gland. In fish, carbonic anhydrase activity has been found in a number of different tissues including: red blood cell (Sanyal *et al.*, 1982; Henry *et al.*, 1988), muscle (Sanyal *et al.*, 1982), swimbladder (Adamson

and Waxman, 1976), and gill epithelium (Dimberg *et al.*, 1988; Henry *et al.*; 1988; Rahim *et al.*, 1988).

Carbonic anhydrase: its roles

In general, CO_2 diffuses faster through solutions if CA is present because CA keeps the concentration gradient high. Studies done by Gros and associates have indicated that CA-catalyzed facilitated diffusion is possibly of great importance in the functioning of the skeletal muscle (Geers and Gros, 1991). Without CA present, the buffering capacity of the $\text{CO}_2\text{-HCO}_3^-$ system cannot be fully utilized because the uncatalyzed reaction is too slow (Thomas, 1983). It appears that there is CA wherever significant amounts of HCO_3^- or H^+ move across cell membrane (Maren, 1987). CA is also involved in intermediary metabolism maintaining the supply of HCO_3^- and H^+ .

In fish, CA is involved in many of the physiological roles of the gills as well as several functions in other tissues. In general, CA has three important functions in fish gills:

1. Acid-base balance; CA supplies the proton for acid excretion at the gills. Lin and Randall (1991) showed that in the presence of acetazolamide, a CA inhibitor, acid excretion was reduced.
2. Ionic regulation; CA provides the counter-ions necessary for the exchange of Na^+ and Cl^- across membranes. Several studies demonstrated a reduction in transepithelial Na^+ or Cl^- uptake after inhibition of branchial CA with acetazolamide (Maetz and Gracia-Romeu, 1964; Kersteller *et al.*, 1970; Kersteller and Kirschner, 1972; Payan *et al.*, 1975; Henry *et al.*, 1988).
3. CO_2 excretion; as in most vertebrates, there is a flux of HCO_3^- into the red blood cell where it is rapidly dehydrated by CA to form CO_2

which diffuses across the respiratory epithelium (Perry, 1986).

The red blood cell of fish

It is generally accepted that the major pathway for CO_2 excretion is HCO_3^- flux through the red cell. In the blood, CO_2 is carried mostly as HCO_3^- but if the blood only relied on the hydration of CO_2 that occurs in the plasma, the amount of CO_2 carried by the blood would be small because the reaction is uncatalyzed in the plasma. CO_2 is hydrated mainly inside the red cell as it diffuses through the blood and HCO_3^- is transferred in the plasma in exchange for Cl^- through the anion exchanger. When the blood arrives at the gills, HCO_3^- enters the red cell in exchange for Cl^- via a band III protein: the anion exchanger. Once inside, HCO_3^- combines with a proton at the catalyzed rate to form CO_2 and H_2O , as describe in the reaction cited above. CO_2 then diffuses out of the cell, through the gill epithelium and into the respiratory water. As CO_2 is excreted, the concentration of HCO_3^- decreases inside the red cell and the influx of HCO_3^- into the red cell is maintained. The protons come from the intracellular buffers and hemoglobin oxygenation. When oxygen binds to hemoglobin, protons are released and used in CO_2 excretion, this phenomena is called the Haldane effect. Fish have a large Haldane effect and consequently large Bohr and Root effect. The Bohr effect is a reduction of hemoglobin-oxygen affinity when protons bind to hemoglobin. The Root effect is a decrease in oxygen carrying capacity of hemoglobin when it binds protons.

Catecholamines released into the blood in stress conditions, for example during extreme hypoxia and exhaustive exercise (see Randall and Perry, 1992), activate a Na^+/H^+

exchanger across the RBC membrane, raising erythrocytic pH (Nikinmaa *et al.*, 1990; Motais *et al.*, 1990). Fish erythrocytes, like those of mammals, are not very permeable to protons (Forster and Steen, 1969). Acid can be transferred between the plasma and the erythrocyte by the cycling of CO_2 and HCO_3^- (Fig. 1). This is known as the Jacob-Stewart cycle. Thus Na^+/H^+ exchange removes protons from the RBC and these will re-enter via the Jacobs-Stewart cycle, the rate limiting step being the uncatalyzed HCO_3^- dehydration reaction in the plasma. As mentioned above, the rise of pH caused by catecholamines will increase hemoglobin-oxygen affinity and as consequence blood will carry more oxygen for the same water Po_2 . pH is not the only factor that influences hemoglobin-oxygen affinity, other ligands such as ATP and more specifically GTP also decrease hemoglobin-oxygen affinity when they bind to hemoglobin.

The problem

Although the major pathway for CO_2 excretion is the HCO_3^- flux through the red cell, some crustaceans use extracellular CA for this purpose (Henry, 1988). In mammals, inhibition of extracellular CA produces a pH disequilibrium but this CA does not seem to be involved in CO_2 excretion (Heming *et al.*, 1986). Since CA does not occur in the plasma of all vertebrates studied to date, this extracellular CA means that some HCO_3^- contained in the plasma has access to CA without going through the red cell. In salmonids, CA is not available to the plasma at the gills. Rahim *et al.* (1988) showed using immunohistology that the basal membrane of the gill epithelium is devoid of CA activity. Henry *et al.* (1988) concluded, from injecting CA inhibitor into intact animals, that there is very little, if any,

membrane-associated CA in the gills.

Why is there this difference between mammals and fish? Why is there no carbonic anhydrase available to fish plasma? Motaïs *et al.* (1989) and Nikinmaa *et al.* (1990) have shown that the addition of CA to the plasma, *in vitro*, will short circuit the action of catecholamines on RBC pH. It has been suggested, therefore, that the absence of CA activity in plasma allows catecholamine regulation of erythrocytic pH. Thus, fish plasma is totally devoid of any CA activity and has erythrocytic pH regulation whereas mammalian plasma has access to CA activity but there is no RBC pH regulation by catecholamines.

Motaïs *et al.* (1989), however, showed that catecholamines could still raise trout RBC pH *in vitro* even in the presence of 0.5 mg/ml CA. These CA levels are an order of magnitude higher than those normally available to mammalian plasma (Effros *et al.*, 1980; Bidani *et al.*, 1983). Low levels of CA activity available to plasma, therefore, may not jeopardize catecholamine induced RBC pH regulation and the absence of CA from fish plasma may be correlated with factors other than RBC pH regulation *per se*.

Fish bodies may consist of more than two thirds muscle. These muscles are predominantly glycolytic and large amounts of acid are released into the blood following burst swimming. In the presence of carbonic anhydrase activity available to the plasma this acid would be rapidly transferred into the RBC and, in addition, the acid would titrate plasma HCO_3^- and be excreted as CO_2 . Both these processes would occur more slowly in the absence of carbonic anhydrase activity available to plasma.

The gills are not only a site for gas exchange and extracellular CA might interfere in the other functions of the gills. When blood reaches the lungs of birds and mammals,

plasma HCO_3^- is transferred into the red cell (in exchange for Cl^-) where it is rapidly dehydrated to CO_2 . CO_2 diffuses across the epithelium following the concentration gradient. In mammals and birds, the CO_2 gradient can be adjusted by changes in ventilation. Thus hyperventilation will increase the CO_2 concentration gradient, and CO_2 excretion will increase; the reverse is also true. Because CO_2 is a weak acid in solution, removal or addition of this molecule will change the pH. Therefore, mammals and birds can alter their acid-base balance via CO_2 excretion. Because oxygen tension is high in air, small changes in ventilation will not affect oxygen uptake. Oxygen concentration is lower in water than in air and water-breathers regulate ventilation for oxygen uptake (Randall and Cameron, 1973). In addition, the solubility of CO_2 in water is about 30 times greater than oxygen. Regulation of ventilation to adjust CO_2 may compromise oxygen uptake in water-breathers (Randall and Cameron, 1973). However, because CO_2 excretion is relatively easy compared with oxygen uptake, CO_2 levels in water-breathers are low. For all those reasons, acid-base balance adjustment by changes in CO_2 excretion would be a bad strategy for water-breathers. In the latter, pH regulation is achieved by changes in plasma HCO_3^- concentration modulated by $\text{Cl}^-/\text{HCO}_3^-$ exchange across the gills (Randall and Cameron, 1973; Janssen and Randall, 1975) and by protons excretion directly via a proton ATPase (Lin and Randall, 1991). Gills are also the major site of osmoregulation compared to lungs which have no function in this matter.

In order to try to answer the question as to why carbonic anhydrase is not available to fish plasma, two series of experiment were undertaken. The first study looked at the possibility that CA available to the plasma in the gills would impair oxygen uptake because

of the accelerated Jacobs-Stewart which would impair the red cell pH regulation and therefore oxygen transport. The second study looked at the possibility of maintaining HCO_3^- stores under acidotic conditions which would be impossible if CA was available to the plasma in the gills.

Figure 1. Jacobs-Stewart cycle in red blood cell

Chapter 2

Carbonic Anhydrase Infusion in Rainbow Trout during Hypoxia

Introduction

The object of experiments reported here was to infuse carbonic anhydrase into trout to obtain activity levels similar to that available to mammalian plasma in order to determine the effect of plasma carbonic anhydrase activity on hemoglobin-oxygen affinity and plasma acid base regulation during exposure to hypoxia. Hypoxia was used as a mechanism for promoting the release of catecholamines and protons into the blood. In addition, hypoxia have been shown to enhance the effect of catecholamines on red cells (Ferguson *et al.*, 1989). The plasma acidosis was the result of anaerobic production of lactic acid in the muscle and the catecholamine induced movement of protons out of the RBC.

Fish subjected to deep hypoxia increase ventilation and release catecholamines. If their oxygen supply does not meet their energy demand, they switch to anaerobic metabolism. It is well known now that if the hypoxia is prolonged, the NTP (ATP + GTP) content of the red cells decreases (see Thomas and Motais, 1990). Although everybody agrees that NTP content is reduced in hypoxic fish, the time course of this decrease varied in different studies [1 or 2 weeks in eel (Wood and Johansen, 1973), 36 hours in carp (Likkeboe and Weber, 1978) and 1 hour in rainbow trout (Tetens and Likkeboe, 1985)]. As mentioned in the general introduction, ATP and more specifically GTP bind to hemoglobin and decrease its affinity for oxygen. It has been concluded that reducing NTP content in the red cells maintains oxygen uptake during hypoxia (Wood and Johansen, 1973).

Materials and Methods

Freshwater rainbow trout [*Oncorhynchus mykiss* (Walbaum)], weighing 300-600 g, were obtained from a local hatchery, and held outdoors at the University of British Columbia, in dechlorinated Vancouver tap water (8-12°C) for at least 2 weeks before experimentation. The animals were fed once a week with commercial trout pellets, but feeding was suspended 3 days prior to surgery. Under MS-222 anesthesia (1:10 000 in NaHCO₃-buffered freshwater), fish were fitted with dorsal aortic catheters according to Soivio *et al.* (1975). Following surgery, fish were allowed to recover for at least 48h in darkened Plexiglass box with recirculating water (8-10°C).

Experimental protocol

For measurements at rest, one blood sample (1.2 ml) was taken from the dorsal aorta and immediately analyzed for plasma pH (pH_e), blood and plasma total CO₂, hematocrit, hemoglobin and oxygen content. The remainder of the blood was centrifuged, plasma was separated from RBC's and both were frozen in liquid nitrogen for further analysis of lactate, catecholamines, adenylates and guanylates and red blood cell pH (pH_i). These fish were not used for the hypoxia experiments because we did not want any stress prior to the infusion and the hypoxia exposure. At the same time that hypoxia was induced (30-35 Torr), one group of fish was infused with saline and another with a solution of carbonic anhydrase to achieve 0.3 g/l in the animal for the duration of hypoxia exposure (6h). Blood samples were taken as described above at 10, 30, 120 and 360 minutes.

Analytical procedures

Plasma pH was determined with a microcapillary electrode (Radiometer G279/G2) coupled to a PHM84 pH meter. Total CO₂ measurements were done using a gas chromatography method (Boutilier *et al.*, 1985) on samples obtained anaerobically. Total CO₂ inside the erythrocytes was obtained from the subtraction of total plasma CO₂ from total blood CO₂, taking into account the hematocrit. Pco₂ was calculated from the Henderson-Hasselbach equation with solubility coefficient and pK calculated according to Boutilier *et al.*, (1984). Hematocrit was measured using microhematocrit tubes centrifuged at 12 000 rpm for 5 minutes. Hemoglobin (Hb) was measured using Drabkin's reagent from Sigma kit (kit #525-A). Mean cellular hemoglobin concentration (MCHC) was calculated as Hb/Hct. The oxygen content of the blood was determined using the method of Tucker (1967). Whole-blood lactate levels were analyzed using the L-lactate dehydrogenase/NAD method (Sigma kit #826-B). High pressure liquid chromatography (HPLC) was used to measure ATP, ADP, AMP, simultaneously with GTP, GDP, GMP. The procedure was carried out using an LKB 2152 HPLC controller and 2150 titanium pump coupled to 2220 recording integrator. The separation was performed on an Aquapore AX-300 7 µm weak anion exchanger (Brownlee laboratories) eluting at 2 ml min⁻¹ at 55°C (Schulte *et al.*, 1992). Analyses of plasma catecholamines levels were performed by HPLC with electrochemical detection, using a Brownlee Spheri-5 reverse-phase column (Technical Marketing, Richmond, BC), a Bioanalytical Systems LC-4A amperometric detector (Mandel Scientific, Rockwood, Ont.) and a Spectra-Physics SP8700 solvent delivery system (Terochem Laboratories Ltd., Edmonton, Alb.), as described by Primmitt *et al.*

(1986). The freeze thaw method of Zeidler and Kim (1977) was used to measure pH_i .

Statistical methods

Statistical significance of data for 6 hour hypoxia was determined using a two-way ANOVA followed by a Dunnet test when comparing the hypoxia values with the resting values and an unpaired t-test when comparing CA infused values with control; both with a statistical significance level of 5%. Data are presented as mean $\pm$ standard error on the mean (SEM).

Results

In resting animals, catecholamines and lactate were similar to resting levels measured by others (Nilsson, 1983; Tetens *et al.*, 1988; Hart *et al.*, 1989; Perry *et al.*, 1989; Thomas and Perry, 1991; McDonald and Milligan, 1992). In both treatments, catecholamines increased significantly after 10 minutes of deep hypoxia reaching a peak at 2h and remained elevated (Table 1). In both groups, lactate was significantly elevated relative to the resting fish at 10 minutes and kept increasing for the next six hours (Fig. 2).

Hypoxia had little effect on plasma pH in saline infused fish (Fig. 3). In the group of fish infused with carbonic anhydrase, however, plasma pH initially increased and then decreased for the next six hours of hypoxic exposure. Erythrocytic pH_i did not vary significantly during hypoxia in either treatment (Fig. 3).

Total CO₂ content of the plasma decreased significantly more in the CA treated animals than in saline treated ones (Fig. 4a). No significant changes were observed in RBC total CO₂ during exposure to hypoxia with or without the infusion of carbonic anhydrase (data not shown). The calculated equilibrium Pco₂ decreased significantly in the CA infused group compared with both normoxic and hypoxic saline infused values (fig. 4b). Pco₂ showed a small decrease in the saline infused group during hypoxia compared with normoxic values which was not significant.

Hematocrit initially increased peaking at 30 minutes of hypoxia (Fig. 5a). Hemoglobin was constant during the first six hours of exposure to hypoxia (Fig. 5b). The ratio of hemoglobin and hematocrit (MCHC), which is an indication of RBC volume, differed between the two groups (Fig. 5c). Initially, hypoxia produced an increase in RBC volume (as indicated by the decrease in MCHC) which was significantly greater than the normoxic values in both treatments. In the saline treatment, RBC volume remained constant for the next six hours. In the CA infused group RBC volume continued to increase and became significantly different from the saline group at 6h. In both treatments, O₂ content dropped dramatically within the first 10 minutes. It then remained constant with no significant difference between the two for the next six hours of hypoxia (fig. 6).

Adenylates and guanylates are expressed as a ratio with hemoglobin to avoid the effect of changes in RBC volume. CA infused fish showed a significant decrease in the total pool of organic phosphates after 30 minutes when compared to resting values (Fig. 7a). In the saline infused group, there is also a decrease which is not significantly different from the resting values. NTP (ATP + GTP) followed the same pattern except that the

concentration at 6h in the CA infused group is significantly different from the saline infused group that is NTP level do not return to resting values in CA infusion.

Table 1. Plasma catecholamine concentrations for saline and carbonic anhydrase (**bold**) infused fish during normoxia and at specific time after hypoxia exposure. Significance of difference from normoxic control: * . (n=7)

	normoxia	hypoxia 10 minutes	hypoxia 30 minutes	hypoxia 120 minutes	hypoxia 360 minutes
adrenaline (ng/ml)	4.15 (1.35)				
Saline		16.2 ± 5.3*	13.2 ± 2.7*	21.6 ± 6.7*	22.4 ± 9.3*
CA		10.2 ± 2.2*	14.1 ± 4.3*	23.1 ± 9.5*	19.3 ± 6.9*
noradrenaline (ng/ml)	3.99 (1.40)				
Saline		22.7 ± 7.8*	17.2 ± 3.8*	25.2 ± 6.2*	17.6 ± 7.9
CA		11.1 ± 2.1*	18.1 ± 6.9*	31.1 ± 15.9*	23.7 ± 10.7

Figure 2. Lactate levels in hypoxic rainbow trout infused with carbonic anhydrase or saline.

* indicates significant difference between hypoxia and normoxia.

Figure 3. Effects of carbonic anhydrase infusion in hypoxic rainbow trout on plasma pH and intracellular pH. * indicates significant difference between hypoxia and normoxia. ** indicates significant difference between saline and carbonic anhydrase infused animals.

Figure 4. Effects of carbonic anhydrase infusion in hypoxic rainbow trout on plasma CO₂ and CO₂ partial pressure. * indicates significant difference between hypoxia and normoxia. ** indicates significant difference between saline and carbonic anhydrase infused animals.

Figure 5. Effects of carbonic anhydrase infusion in hypoxic rainbow trout on hematocrit, hemoglobin concentration and mean cell hemoglobin concentration. * indicates significant difference between hypoxia and normoxia. ** indicates significant difference between saline and carbonic anhydrase infused animals.

Figure 6. Effects of carbonic anhydrase infusion in hypoxic rainbow trout on oxygen content. * indicates significant difference between hypoxia and normoxia.

Figure 7. Effects of carbonic anhydrase infusion in hypoxic rainbow trout on total organic phosphates and nucleotide triphosphates. * indicates significant difference between hypoxia and normoxia. ** indicates significant difference between saline and carbonic anhydrase infused animals.

Discussion

Carbonic anhydrase was infused continuously during the six hours of hypoxia and the calculated concentration of CA in the plasma, assuming no loss, was nearly an order of magnitude higher than that available to plasma in the mammalian lung (Effros *et al.*, 1980). Lessard *et al.* (1993) showed that the concentration of CA was still 60% of the initial values after 60 minutes of a bolus injection indicating a slow rate of removal of CA activity from the plasma. Thus the plasma CA activity, assuming some loss, was probably similar to that available to plasma flowing through the mammalian lung. This activity of CA was insufficient to affect the regulatory effects of catecholamines on RBC pH as there was no difference in either RBC pH or blood oxygen content between the saline and CA infused groups during the first six hours of hypoxic exposure (Fig. 3 and 6). The fact that there was a catecholamine effect is indicated by the marked increase in RBC volume in both groups (Fig. 5c). These results are in contrast to the *in vitro* experiments of Nikinmaa *et al.*, (1990) who added 3g/l (rather than 0.3g/l) CA to the blood and short-circuited the effects of catecholamines on RBC pH. Motaïs *et al.* (1989), also *in vitro*, used a lower concentration (0.5g/l) than Nikinmaa, but similar to ours, and observed an increase of pH_i in response to the addition of catecholamines. So it seems that to short-circuit the rise in pH_i the concentration of CA needed is higher than that used in this study and, therefore, that available to mammalian plasma in the lungs. However, pH_e has a very different behaviour at the onset of hypoxia between saline and CA infused animals which make it difficult to interpret the regulatory effects of catecholamines on pH_i . Nevertheless, oxygen

content was similar in both groups indicating a comparable hemoglobin-oxygen affinity. Lastly, the enzyme carbonic anhydrase has one of the highest turnover numbers and for that reason the concentration of CA might not be the limiting factor. The difference in the results between the study of Nikinmaa *et al.* (1990) and Motaïs *et al.* (1989) and this study could be due to different experimental conditions, especially plasma buffering, which would affect catalyzed HCO_3^- dehydration rates.

The increase in CA activity in fish plasma in these experiments had a marked effect on plasma total CO_2 content. PaCO_2 in the saline infused fish is determined by the rate of $\text{Cl}^-/\text{HCO}_3^-$ exchange across the RBC membrane and ventilation (Perry, 1986). In the CA infused group PaCO_2 will be determined largely by ventilation because plasma HCO_3^- dehydration is catalyzed and does not have to pass through the anion exchanger to be excreted. The end result is that the equilibrium PaCO_2 level is higher in the saline infused fish compared with the CA infused fish (Fig. 4). Hypoxic exposure results in an increase in ventilation and therefore a small reduction in PaCO_2 (Thomas *et al.*, 1988), which contributes to the initial reduction in total CO_2 observed in fish exposed to hypoxia (Fig. 4). The subsequent decrease in total CO_2 , especially in CA infused fish, is probably related to acid titration of plasma HCO_3^- . The increase in plasma lactate concentration during hypoxia was the same in both saline and CA infused groups (Fig. 2), indicating that approximately the same amount of metabolic acid was produced by both groups. Blood total CO_2 decreased more rapidly in the CA, compared with the saline infused group (Fig. 4), presumably because plasma HCO_3^- is titrated to a greater extent by the acid released by anaerobic metabolism in the carbonic anhydrase infused animals. CA infusion caused

a rapid removal of protons via HCO_3^- dehydration. This resulted in a marked alkalosis not seen in the saline infused group. Much of the changes in HCO_3^- concentration was in the first 30 minutes of hypoxia and infusion, but the subsequent rate of change in HCO_3^- concentration was much the same in both groups for the next six hours indicating that CA was no longer the determining factor. During this time blood pH in saline infused fish remained constant, that is proton entry into the blood was the same as proton excretion. In the CA infused animals blood pH fell after the initial alkalosis, *i.e.* in the blood, proton entry exceeded proton excretion at the gills. It is possible, therefore, that CA infusion may have resulted in increased acid removal from muscle.

Red cell volume increased upon catecholamine stimulation in both groups (Fig. 5c). This can be accounted for by the entry of ions, mainly Na^+ and Cl^- , with water following osmotically (Fievet *et al.*, 1988; Baroin *et al.*, 1984). CA infusion, however, caused a further increase in RBC volume after 30 minutes exposure to hypoxia. This could be due to increased activity of the Na^+/H^+ exchanger, which has been shown to be enhanced by a fall in blood pH (Nikinmaa *et al.*, 1990) like that seen following CA infusion in hypoxic fish (Fig. 3). The levels of organic phosphates remained depressed in the CA infused group (fig. 7) probably because of increased ATP utilization by the Na^+/H^+ exchanger. RBC swelling resulted in the initial increase in hematocrit observed when fish were exposed to hypoxia, any release of red blood cells from the spleen was offset by RBC removal during sampling.

Why then is there no carbonic anhydrase activity available to blood plasma in fish? It is not simply to permit catecholamine induced pH regulation of the red blood cell because this occurs in the presence of low levels of CA. In general, the absence of CA

activity reduces the titration of HCO_3^- when protons are liberated from muscle into the blood. The red blood cells are protected from this acidosis because the acid is transferred to the RBC more slowly than it would be if CA was present. In addition, the transfer of acid across the gill epithelium is not short-circuited by a rapid HCO_3^- hydration/dehydration reaction in the plasma causing a rapid cycling of CO_2 between water and plasma. The uncatalyzed $\text{CO}_2/\text{HCO}_3^-$ reaction in the plasma, therefore, facilitates blood pH regulation due to acid transfer across the gill epithelium.

Fish tolerate a plasma acidosis but protect the RBC from pH changes and maintain oxygen transfer by having no CA activity available to the plasma. This also prevents large changes in plasma HCO_3^- and allows the animal to adjust plasma HCO_3^- levels in order to regulate pH. This is important because fish do not regulate pH by changes in PaCO_2 via ventilation. Mammals adjust PaCO_2 via ventilation and have carbonic anhydrase activity available to the plasma as it flows through the lungs.

Chapter 3

Carbonic Anhydrase Infusion in Rainbow Trout Following Exhaustive Exercise

Introduction

In the study presented in chapter 2, it was thought that CA would short-circuit the pH regulation of the red blood cells by catecholamines and as a result would impair oxygen uptake. CA in plasma did not cause the expected reduction in blood oxygen content during hypoxia but did have a marked effect on plasma total CO_2 and pH_e . The CO_2 decrease was explained by the titration of HCO_3^- by protons released during anaerobic metabolism and the adrenergic extrusion from the red cells and the subsequent excretion of molecular CO_2 at the gills. It was concluded that the decrease in pH_e following CA infusion was due to an imbalance in acid excretion at the gills and acid load at the tissues. So CA infusion changes the movement of acid in the animal.

In chapter 2, it was reported that total CO_2 content of the plasma decreased by about 60% of the resting value during hypoxia. Thus, if CA is available to the plasma, the store of HCO_3^- is rapidly depleted in acidotic conditions. In the light of this finding, the present experiment was conducted to see if, in fact, the HCO_3^- reserve would be depleted with a different pattern of acidosis. In hypoxia, the acid is released slowly during anaerobic metabolism. If it is true that the greater decrease in total CO_2 following CA infusion during hypoxia was due to the HCO_3^- titration, then, the HCO_3^- decrease would be more drastic if the acid should be released in a shorter time as in recovery after exhaustive exercise. Furthermore, exhaustive exercise promotes the release of catecholamines and an increase in ventilation as in deep hypoxia.

To further investigate the fact that CA is not available to the plasma in fish gills, we

infused CA during the recovery from exhaustive exercise and monitored the acid-base balance and oxygen transport in the blood of trout.

Materials and Methods

Freshwater rainbow trout [*Oncorhynchus mykiss* (Walbaum)], weighing 700-850 g, were obtained from a local hatchery, and held outdoors at the University of British Columbia, in dechlorinated Vancouver tap water (6-7°C) for at least 2 weeks before experimentation. The animals were fed once a week with commercial trout pellets, but feeding was suspended 3 days prior to surgery. Under MS-222 anesthesia (1:10 000 in NaHCO₃-buffered freshwater), fish were fitted with dorsal aortic catheters according to Sovio *et al.* (1975). Following surgery, fish were allowed to recover for at least 48h in a Brett type respirometer with recirculating water (6-7°C).

Experimental protocol

The animal was swim to exhaustion by gradually increasing the speed of the water in a swim tunnel. At exhaustion, the animal was transferred to a dark Plexiglass box and a blood sample (800 µl) was immediately taken from the dorsal aorta. Plasma pH (pH_e), plasma total CO₂, hematocrit, hemoglobin, oxygen content and oxygen partial pressure (Po₂) were immediately analyzed. The remainder of the blood was centrifuged, plasma was separated from RBC's and both were frozen in liquid nitrogen for further analysis of lactate, catecholamines and red blood cell pH (pH_i). Just before exhaustion, one group of

fish was injected with 1ml/kg of saline and another with 1ml/kg of carbonic anhydrase solution of 10 000 units. After the fish was transferred in the Plexiglass box, saline or CA was continuously infused for the duration of the experiment (6h). Blood samples were taken as described above at 30, 60, 120 and 360 minutes of the recovery period.

Analytical procedures

The procedures used for the present experiment were the same as those described in chapter 2.

Statistical methods

Statistical significance of data was determined using a two-way ANOVA followed by a Dunnet test when comparing the hypoxia values with the resting values and an unpaired t-test; both with a statistical significance level of 5%. Data are presented as mean $\pm$ standard error on the mean (SEM).

Results

Catecholamines were released before or at exhaustion as they were initially high and slowly decrease thereafter. CA infusion had no effect on catecholamines release (table 2). At zero time, immediately after exercise, lactate was above resting levels (see chapter 2), peaked at 2 hours and then decreased. There was no significant difference between saline and CA infused animals (fig. 8).

Hematocrit was initially very high and slowly decreased for the rest of the experiment (Fig. 9c). Hemoglobin was also high at the beginning of the recovery period and showed a steady decrease reflecting the hematocrit (Fig. 9b). A significant difference was not observed in these parameters between treatments (fig. 9a-b). The ratio of hemoglobin and hematocrit (MCHC), which is an indication of RBC volume, was initially low (indicating an increase in volume) and increased to return to resting values at 6 hours (Fig. 9a).

The amount of oxygen bound to hemoglobin (O_2/Hb) increased slightly right after exhaustion and remained high for the rest of the recovery period (Fig. 10a). CA infused fish had a similar pattern to the saline infused group. Oxygen partial pressure was higher in control fish than in CA infused fish and there was a significant difference at 60 minutes (Fig. 10b). By 2 hours, Po_2 in the CA infused group had reached the control values and there was no significant difference thereafter (fig. 10b).

As expected, plasma pH dropped below resting levels at the onset of recovery (Fig. 11). In the saline infused group the drop was greater than in the CA infused group and the difference was significant at time zero. Then, pH_e recovered at the same rate in the two groups but since pH_e in the control group was lower at the beginning of the recovery period, it was always lower than in the CA infused group. In the latter group, pH_e overshoot the resting levels and was significantly higher than the saline infused group. On the other hand, pH_i did not vary significantly during the recovery period in either treatment (fig. 11).

Total CO₂ content of the plasma decreased significantly more in the saline treated animals than in CA treated ones (Fig. 12a). In the CA infused fish, total CO₂ decreased as well but was significantly different from the saline infused group at 1 and 2 hours. After 2 hours total CO₂ increased to reach the time zero values and there no significant difference between the two groups (fig. 12a). The calculated equilibrium Pco₂ was significantly different in the CA infused fish compared with the sham right at the beginning of the recovery period (Fig. 12b). In the saline infused fish, Pco₂ was more than twice higher than the resting values (see chapter 2, fig. 4). At thirty minutes, Pco₂ levels were similar in the two groups, 2-3 torrs.

Table 2. Plasma catecholamine concentrations for saline and carbonic anhydrase (bold) infused fish during recovery after exhaustive exercise. Significance of difference from time zero after exhaustive exercise: * .

	0 minutes	30 minutes	60 minutes	120 minutes	360 minutes
adrenaline (ng/ml)					
Saline	64.4 ± 39.9	10.8 ± 4.5	23.9 ± 12.9	8.9 ± 2.9	27.1 ± 17.2
CA	35.9 ± 11.9	12.5 ± 3.1*	16.9 ± 5.1*	13.4 ± 5.2*	31.9 ± 17.5
noradrenaline (ng/ml)					
Saline	8.1 ± 2.9	1.5 ± 0.5*	2.3 ± 1.3*	1.2 ± 0.5*	2.3 ± 1.5*
CA	6.6 ± 1.4	1.9 ± 0.5*	2.3 ± 0.7*	1.9 ± 0.7*	2.8 ± 1.4*

Figure 8. Lactate levels in rainbow trout infused with carbonic anhydrase or saline following exhaustive exercise. * indicates significant difference between hypoxia and normoxia.

Figure 9. Effects of carbonic anhydrase infusion in rainbow trout during recovery after exhaustive exercise on hematocrit, hemoglobin concentration and mean cell hemoglobin concentration. * indicates significant difference between hypoxia and normoxia.

Figure 10. Effects of carbonic anhydrase infusion in rainbow trout during recovery after exhaustive exercise on the amount of oxygen bound to hemoglobin and oxygen partial pressure of arterial blood. * indicates significant difference between hypoxia and normoxia. ** indicates significant difference between saline and carbonic anhydrase infused animals.

Figure 11. Effects of carbonic anhydrase infusion in rainbow trout during recovery after exhaustive exercise on plasma pH and intracellular pH. * indicates significant difference between hypoxia and normoxia. ** indicates significant difference between saline and carbonic anhydrase infused animals.

Figure 12. Effects of carbonic anhydrase infusion in rainbow trout during recovery after exhaustive exercise on plasma CO₂ and CO₂ partial pressure. * indicates significant difference between hypoxia and normoxia. ** indicates significant difference between saline and carbonic anhydrase infused animals.

Discussion

RBC volume increased upon catecholamine stimulation in both experimental groups. The increase was slightly, though not significantly greater, in the CA infused animals. These findings are in accordance with the ones presented in chapter 2. As expected, RBC volume returned to resting levels by 6 hours since catecholamine concentrations decreased and the pH_e increased close to resting values (see chapter 2). Hematocrit was very high at the beginning of the recovery period probably due to RBC recruitment from the spleen and RBC swelling. It then decreased to resting levels due to RBC shrinkage and sampling.

During the entire recovery period CA had no effect on the amount of oxygen bound to hemoglobin. This was also observed in the other study presented in the preceding chapter where the oxygen content of the CA infused fish was similar to the control group. A lower Po_2 was observed in the first hour of the recovery period in CA infused animals. This could result from a disequilibrium in the CO_2 hydration/dehydration reaction which is normally uncatalyzed in the plasma. As the blood flows through the gills, CO_2 is excreted and CO_2 hydration is enhanced. As the blood flows away from the gills, molecular CO_2 is formed and trapped in the blood. CO_2 then enters the RBC and is rapidly dehydrated by CA present inside the cell. The protons produced from this dehydration reaction bind to hemoglobin and dislodge the oxygen from hemoglobin, thus increasing Po_2 . In the presence of CA, CO_2 formation is accelerated and the higher Po_2 is not observed because the CO_2 hydration/dehydration reaction is at equilibrium at the gills. In both groups, PaO_2 and HbO_2 tended to be low immediately following exercise. Primmitt *et al.*

(1986) observed a decrease in gill ventilation frequency associated with exhaustion. The frequency of gill ventilation was not measured in the present experiment but a decrease in this parameter could reduce the oxygen content of the blood.

In the saline infused animals, CO_2 tension at equilibrium immediately following burst swimming was higher than in resting animals (fig. 4 and fig. 10). Pco_2 was similar between the two groups for the rest of the recovery period. This high Pco_2 without a corresponding decrease in PaO_2 has been observed in many other studies (Turner *et al.*, 1983; Milligan and Wood, 1986; McDonald *et al.*, 1989). The Pco_2 presented here were calculated reflecting equilibrium values and are probably not tensions that animals are experiencing. Thomas and Perry (1991) showed that there is still an increase in Pco_2 after exhaustive exercise while recording continuously using an extracorporeal loop. It has been postulated that adrenergic stimulation reversed the Pco_2 gradient between red cells and plasma (CO_2 entering the cells under those conditions) and that the red cells played a small role in CO_2 excretion when catecholamines are present (Wood and Perry, 1991). This would lead to an increase in Pco_2 despite the hyperventilation. In addition, Primmitt *et al.* (1986) reported that ventilation was reduced immediately after exhaustive exercise. Since catecholamines were present after exhaustive exercise, ventilatory adjustments could explain the Pco_2 increase in the saline infused animals. In CA infused animals, Pco_2 at equilibrium decreased, probably because CO_2 excretion does not have to go through the red cells when CA is present in the plasma. Perry and Wood (1989) reported that the respiratory acidosis was reduced after exhaustive exercise in the presence of CA, supporting the results presented here.

If the argument that acid loading titrate HCO_3^- to form CO_2 , which is excreted at the gills, is true, then HCO_3^- should have decreased more in the CA animals than in the control animals. This is not the case as CO_2 content in the plasma was higher in the CA infused fish than in the controls. Plasma pH was also higher in CA infused animals than in saline infused fish. We can presume that muscle acidification was the same in both groups of animals because plasma lactate concentrations were similar. If the acidification of the muscles was the same, then either the acid was retained in the muscle or acid is excreted at a higher rate across the gills. It is more likely that acid was retained in the muscles when CA was present in the plasma because there were no limitation for HCO_3^- dehydration in the gills. In other words, CO_2 and acid equivalents can be excreted faster because of the presence of CA. This could not be the case because if acid was excreted at a higher rate to raise the pH_e then total CO_2 levels would be lower in the CA infused animals than in the saline ones. This difference in total CO_2 would result from the titration of HCO_3^- under acidotic conditions. It appears as though CA reduces the acid transfer in the plasma from the muscle. The mechanisms of this retention are yet to be discovered.

Chapter 4

General Discussion

Carbonic anhydrase did not have the expected effects in both studies. In hypoxia, CA did not short-circuit the regulatory effects of catecholamines on red blood cell pH thus maintaining Hb-O₂ affinity. CA infusion, however, greatly reduced HCO₃⁻ concentrations in the plasma and created an acidosis which could not be explained by CO₂ removal or addition. The hypothesis of the second study was that in the face of rapid acid excretion (recovery after exhaustive exercise compared with deep hypoxia) HCO₃⁻ stores would be rapidly depleted. The opposite was observed; HCO₃⁻ stores in the CA infused animals were always higher than in the saline infused animals. Obviously, CA in the plasma changed the rate at which acid is transferred within and/or excreted by the body.

The patterns of acid formation were different in the two studies. During hypoxia, CO₂ production probably remained the same or maybe might have even decreased while acid production increased due to anaerobic metabolism. After exhaustive exercise, CO₂ production and acid release from the muscle, were greatly increased. CO₂ loading in the blood from the muscle would be enhanced by CA in the plasma because the gradient from the muscle to the blood would be kept lower. That is to say that as soon as CO₂ is transferred to the blood it is hydrated to form HCO₃⁻, maintaining the Pco₂ gradient. This in turn will lower the blood pH and might decrease acid excretion from the muscle. The inverse may also be true: H⁺ are released in the blood, shifting the reaction to CO₂ formation and decreasing the gradient for CO₂ excretion in the blood. At the gills one can imagine the same interaction: one product of excretion limiting the excretion of the other. CA available to the plasma at the gill level might inhibit acid excretion or osmoregulation by altering the turnover of protons and/or CO₂. Fish are often exposed to acid waters,

especially in freshwater. CA present in the plasma could potentially enhance acid uptake by cycling CO_2 while maintaining the transepithelial acid gradient (fig 13). The same thing would probably happen if the fish was submitted to an hypercapnic environment: a larger transfer of CO_2 in the blood due to CA that maintains the gradient. In the lungs, these situations do not appear since lungs do not play any function in excretion of proton across the membrane nor in ion regulation.

The reasons why CA is not available to the plasma at the gills could not be determined by the two studies presented herein. In the presence of CA activity, there is probably reduced HCO_3^- flux through the red blood cell during the oxygenation and deoxygenation process. HCO_3^- dehydration consumes the protons produced by hemoglobin oxygenation. A reduced HCO_3^- flux through the red cell will result in larger oscillations in erythrocyte pH during oxygenation of the hemoglobin and may impair blood oxygen saturation in fish with a marked Root shift. This does not happen in hypoxic conditions because of the reduced hemoglobin-oxygen saturation. The hypoxia was severe and the pH might not affect the oxygen binding at such low saturation of the hemoglobin. The α -chains of hemoglobin are acetylated in fish and therefore do not bind protons (Jensen, 1989). The β -chains do bind protons and they are also the two last chains to bind oxygen in the oxygenation process. Therefore, at the level of hypoxia at which the experiment was done might be too low to detect any difference in oxygenation between saline and CA infused animals. During recovery from exhaustive exercise, impaired oxygenation might have been the case as the oxygen stores in the first minutes of recovery were lower, although not significantly, in the CA infused fish.

The question remains as to why is there no carbonic anhydrase available to fish plasma? The reason could be phylogenetic; that is, membrane bound CA did not occur in the evolution of fish. Since we know so little about the location and distribution of CA in lower vertebrates like fish we cannot rule out this possibility. CA infusion did not drastically impair oxygenation under all experimental conditions. In addition, it did not deplete HCO_3^- stores in all acidotic conditions. CA infusion, however, did change the pattern of acid loading at the muscle and, or, acid excretion at the gills. Fish plasma is not very well buffered and a large Haldane effect is an important contribution to buffering pH effects associated with CO_2 transport. The evolution of a large Haldane effect could be associated with large Bohr and Root effects. That is these effects may be structurally linked together in the hemoglobin molecule. It is not clear whether selective forces have operated on the Haldane, Bohr or Root effects. Independent of the mechanism of evolution, the result will lead to a large release of protons during oxygenation. If the animal wants to maintain its oxygen carrying capacity, those protons have to be excreted or buffered. Fish hemoglobin has a low buffering capacity when compared to other vertebrates (Jensen, 1989) so the protons released upon oxygenation have to be removed in order to achieve full saturation of hemoglobin. HCO_3^- flux through the red cell ensures that the protons are excreted as CO_2 and cannot bind again to hemoglobin. The absence of CA in the plasma ensures that HCO_3^- flux through the red cell is maintained.

The results of these two studies do not allow us to distinguish between enhancement or inhibition of acid excretion at the gills or at the muscles. Further experiments are needed to determine where the acid is going during CA infusion.

Figure 13. Jacobs-Stewart cycle in the gills

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