TEMPERATURE-RELATED CHANGES IN THE ERYTHROCYTIC CARBONIC ANHYDRASE (ACETAZOLAMIDE-SENSITIVE ESTERASE) ACTIVITY OF GOLDFISH, *CARASSIUS AURATUS*

BY ARTHUR H. HOUSTON AND KAREN M. MEAROW

Department of Biological Sciences, Brock University, St Catharines, Ontario, Canada, L2S 3A1

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SUMMARY

1. Carbonic anhydrase activity in 'membrane' and 'cytosol' fractions of goldfish erythrocytes was assayed by the p-nitrophenyl acetate procedure following thermal acclimation.

2. The thermal sensitivity of 'membrane'-associated activity was apparently unaltered by acclimation. 'Cytosol' activity in warm-acclimated specimens was somewhat more thermosensitive than that of animals maintained at low temperature.

3. Significant increases in specific activity, and activity per unit volume of packed cells and blood were observed at higher temperatures when assays were conducted at the temperatures at which the system actually functions in the fish. By contrast, when determinations were carried out at a standard temperature (41°C) corresponding to the upper incipient lethal for this species, activity was either unaffected, or declined as acclimation temperatures increased.

4. Changes in carbonic anhydrase activity following acclimation are consistent with the hypothesis that this system is implicated in the maintenance of stable plasma chloride levels, and the suggestion that alterations in red cell chloride levels with temperature are, in part at least, attributable to concomitant variations in enzyme activity.

INTRODUCTION

The involvement of carbonic anhydrase (EC 4.2.1.1) in the regulation of sodium and chloride balance by freshwater teleosts has been well documented (Maetz, 1974). Recent studies suggest that the carbonic anhydrase system of the red cell may be of particular importance in at least two features of adaptive ionoregulatory response to increases in environmental temperature. Kerstetter & Kirschner (1972) have hypothesized that chloride uptake by the rainbow trout *Salmo gairdneri* is largely dependent upon a HCO₃⁻:Cl⁻ exchange mechanism driven by blood-generated bicarbonate ion. Consistent with this is the observation that acclimation of trout to higher temperatures is accompanied by substantial increases in the carbonic anhydrase activity of blood,
but not of gill or kidney (Houston & McCarty, 1978). It has also been demonstrated (Smeda & Houston, 1979) that increases in erythrocytic carbonic anhydrase activity can be correlated with the rise in red cell chloride which takes place in rainbow trout acclimated to increased temperatures. In mammalian systems chloride mimics 2,3-DPG and ATP in negatively modulating haemoglobin-oxygen affinity (de Bruin et al. 1974; Rollema et al. 1975). Thus, increases in erythrocytic chloride may facilitate oxygen release at the cellular level, and would be of some adaptive value in the satisfaction of temperature-induced increases in oxygen demand.

More eurythermal species such as the carp *Cyprinus carpio* and goldfish *Carassius auratus* must satisfy proportionally larger increases in oxygen demand than the salmonids over their more extended thermal tolerance zones and must do so, moreover, in the face of larger reductions in oxygen availability (Beamish, 1964). Compared to the trout, both exhibit substantially greater changes in red cell chloride content with temperature (Catlett & Millich, 1976; Houston & Smeda, 1979). It was of interest, therefore, to evaluate the effects of temperature upon erythrocytic carbonic anhydrase in a eurythermal teleost, and this study reports information for goldfish acclimated to 5, 15, 25 and 35 °C.

**MATERIALS AND METHODS**

*Origin and maintenance of experimental animals*

Goldfish ranging in weight from 10.2 to 35.6 g (mean: 18.3 g, standard error: 0.70 g) were obtained from a local commercial supplier, and held in the laboratory in 200 l insulated fibreglass aquaria at a density of approximately 1–2 g l⁻¹. Tank water was circulated at ~ 5 l min⁻¹ through activated charcoal-glass wool filters. Each tank was equipped with a photoperiod hood, and all test groups were maintained on a 12 h light:12 h darkness photoperiod regime throughout acclimation periods of not less than 3 weeks. Thermistor-equipped regulators of local design and construction (J. Rustenberg, unpublished) linked to auxiliary heating and refrigeration units were used to hold water temperatures within ±0.5 °C of desired values.

The dechlorinated St Catharines city water used in the study is of moderate total hardness (135–142 mg l⁻¹, as CaCO₃) and alkalinity (91–96 mg l⁻¹, as CaCO₃) and ranges in pH from 7.4 to 7.8. Oxygen concentrations varied inversely with temperature, but exceeded 75–80% saturation.

Specimens were fed once daily, *ad libitum*, on a commercial ration and, as judged from general activity, feeding behaviour and the absence of overt disease symptoms, remained in healthy condition throughout the study period.

*Sample preparation*

Specimens were stunned and blood samples drawn by caudal vessel puncture into ammonium heparin-treated syringes. Microhaematocrit determinations were immediately carried out in triplicate, and on the basis of these duplicate preparations containing sufficient whole blood to yield 0.125 ml of packed cells prepared. These were centrifuged (5000g, 5 min, 2 °C) and plasma withdrawn. Cells were washed 4 times in 0.7% saline with intervening centrifugations (5000g, 1 min, 2 °C), and after the
Carbonic anhydrase activity of goldfish

Final wash resuspended in 200 μl of saline. Double glass-distilled water was added to a final volume of 5.0 ml, and the preparation vortexed (4.0 min) to promote haemolysis.

Crude separations of ‘membrane’ and ‘cytosol’-sited activities were carried out by centrifugation of lysed samples (30000g, 30 min, 2 °C). The resulting pellet was separated from the supernatant and washed 3 times with 2.5 ml aliquots of distilled water. Wash waters were assayed for carbonic anhydrase activity, but this was only detected occasionally (and barely) in the first wash. Washed pellets were resuspended in 2.5 ml of distilled water and vortexed to transparency. A final centrifugation (3000g, 5 min, 2 °C) was carried out to remove bubbles introduced during the preceding step, but neither this nor more prolonged centrifugations (up to 45 min) led to pellet formation. Only very finely fragmented material was seen by high-resolution, phase-contrast microscopy. In view of ultrastructural studies upon mature teleostean erythrocytes (Iuchi, 1973) it seems probable that much of the pellet consisted of membrane fragments, and that activities subsequently detected reflected the action of membrane-sited enzyme.

Centrifugation of the supernatant (100000g, 45 min, 2 °C) failed to produce further sedimentation or to alter activity, and the results of microscopic examination were negative. Activities obtained with these preparations were therefore interpreted as evidence of free, or weakly-bound enzyme.

Assay procedure

The β-nitrophenyl acetate procedure was used with modifications described earlier (Houston & McCarty, 1978) to assay ‘membrane’ and ‘cytosol’ preparations at the appropriate acclimation temperature (5, 15, 25 or 35 °C) and at 41 °C, a temperature approximating the upper incipient lethal for this species. As in previous studies (McCarty & Houston, 1977; Houston & McCarty, 1978; Smeda & Houston, 1979) this procedure was adopted to provide a basis for relating activities at a physiologically realistic maximum temperature to those at the temperatures at which the system actually operates. In addition, samples from fish acclimated to 5 and 35 °C were assayed at 5, 15, 25, 35 and 45 °C to examine acclimatory effects upon the thermal sensitivities of ‘membrane’ and ‘cytosol’ activities.

Reactions were initiated by mixing 1.0 ml of 3 mM β-nitrophenyl acetate substrate with a preparation preincubated at the appropriate temperature. This was promptly introduced into the thermostatted microflowthrough cell of a Bausch and Lomb Spectronic-700 and absorbance monitored at 348 nm for a 2 min period using a Fisher Omniscribe recorder. Duplicate assays were first carried out to establish total esterase activity, and then repeated in duplicate with fresh acetazolamide-inhibited preparations. The difference in mean rate was taken as a measure of carbonic anhydrase activity, appreciating that the term ‘acetazolamide-sensitive esterase’ activity is technically more appropriate.

Activity values have been reported in three ways: specific activity (μM min⁻¹ g⁻¹, non-haemoglobin protein), activity per unit volume of packed cells (μM min⁻¹ ml⁻¹, packed erythrocytes) and activity per unit volume of blood (μM min⁻¹ ml⁻¹, whole blood). In the case of specific activity determinations total protein was estimated by the modified Schacterle–Pollack modification of the Lowry procedure (Albro, 1975),
and corrected for haemoglobin as determined by the alkaline haematin method (Anthony, 1961).

Statistical analysis

Comparisons were based upon use of single-classification analysis of variance. All data were subjected to base-10 logarithmic or arc-sin transformation as appropriate before analysis, and significance attributed to differences at the 0.05 level or better.

RESULTS

Carbonic anhydrase activity in warm- and cold-acclimated goldfish

Mean specific activities of 'membrane' and 'cytosol' preparations from 5 and 35 °C specimens incubated at temperatures ranging from 5 to 45 °C are indicated in Fig. 1. In both groups specific activity increased with incubation temperature although, as indicated by $Q_{10}$ values, the effect of temperature tended to decrease at higher...
Carbonic anhydrase activity of goldfish

Table 1. 'Membrane' and 'cytosol' protein content, specific activities at acclimation temperature and 41 °C, Q_{10} values and thermal coefficients in thermally acclimated goldfish

(Values reported as mean ± one standard error of the mean, N = 10–12.)

<table>
<thead>
<tr>
<th>Acclimation temperature (°C)</th>
<th>Protein (mg ml⁻¹) packed cells</th>
<th>Specific activity (µM min⁻¹ g⁻¹)</th>
<th>Thermal Q_{10} coeff.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(1) Acclimation temperature</td>
<td>(a) 41 °C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:2</td>
<td></td>
</tr>
<tr>
<td>'Membrane' preparations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 28.8 ± 12.52</td>
<td>10.2 ± 1.51</td>
<td>54.3 ± 6.69</td>
<td>0.18</td>
</tr>
<tr>
<td>15 43.3 ± 5.58</td>
<td>10.6 ± 1.60</td>
<td>33.2 ± 3.89</td>
<td>0.31</td>
</tr>
<tr>
<td>25 31.0 ± 4.55</td>
<td>19.6 ± 1.64</td>
<td>36.5 ± 4.36</td>
<td>0.53</td>
</tr>
<tr>
<td>35 30.5 ± 4.44</td>
<td>31.0 ± 3.55</td>
<td>37.6 ± 3.97</td>
<td>0.82</td>
</tr>
<tr>
<td>'Cytosol' preparations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 74.8 ± 19.66</td>
<td>5.5 ± 0.89</td>
<td>28.4 ± 3.92</td>
<td>0.19</td>
</tr>
<tr>
<td>15 107.7 ± 10.74</td>
<td>8.4 ± 1.17</td>
<td>29.0 ± 2.41</td>
<td>0.28</td>
</tr>
<tr>
<td>25 100.4 ± 6.84</td>
<td>16.1 ± 1.76</td>
<td>30.2 ± 3.89</td>
<td>0.53</td>
</tr>
<tr>
<td>35 77.9 ± 10.33</td>
<td>18.5 ± 2.32</td>
<td>29.0 ± 3.75</td>
<td>0.68</td>
</tr>
</tbody>
</table>

temperatures. No significant differences in either 'membrane' or 'cytosol' activities were encountered. In the case of 'cytosol' preparations, however, Q_{10} values for the 35 °C group were consistently higher than those of cold-acclimated animals. This proved to be significant by rank analysis, suggesting that this form of carbonic anhydrase activity may have been influenced by the acclimation process. On the other hand, Arrhenius plots provided little evidence of any significant variation attributable to acclimation in either fraction.

Specific activity

Table 1 summarizes observations upon the specific activities of 'membrane' and 'cytosol' preparations. As was the case in rainbow trout (Smeda & Houston, 1979), the non-haemoglobin protein content of 'cytosol' preparations varied with acclimation temperature, being significantly higher (P < 0.05) at the intermediate than at the extreme temperatures. No significant differences were apparent in 'membrane' fractions. 'Membrane' specific activity, as assayed at 41 °C, fell significantly (P < 0.05) between 5 and 15 °C. However, the activities of 'membrane' preparations incubated at acclimation temperature - a more physiologically meaningful measure of activity than the preceding one - increased substantially, and significantly (P < 0.01) with temperature; an approximately threefold increase taking place between 5 and 35 °C. If it is assumed that activity at 41 °C is a reflection of the physiological maximum, the increase in relative activity was 4.39 with temperature between 5 and 35 °C. Mean Q_{10} values (1.21–1.59) varied inversely with temperature, and were generally consistent with previously reported Q_{10} estimates for this enzyme (Davis, 1961). Comparable coefficients relating activities obtained under in vivo incubation temperature conditions (properly referred to as 'thermal coefficients' rather than Q_{10} values) were, with one exception (15 °C/5 °C–1.04), of similar magnitude.

Unlike 'membrane' activity, 'cytosol' activity did not change significantly with
Table 2. Carbonic anhydrase activities (μm min⁻¹ ml⁻¹, packed cells) in ‘membrane’ and ‘cytosol’ fractions of the red blood cells of thermally acclimated goldfish

(Values reported as mean ± one standard error of the mean. N = 10-12.)

<table>
<thead>
<tr>
<th>Acclimation temperature (°C)</th>
<th>'Membrane' preparations</th>
<th>'Cytosol' preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(1) Acclimation temperature</td>
<td>(2) 41 °C</td>
</tr>
<tr>
<td>5</td>
<td>0.26 ± 0.021</td>
<td>1.43 ± 0.201</td>
</tr>
<tr>
<td>15</td>
<td>0.47 ± 0.059</td>
<td>1.16 ± 0.110</td>
</tr>
<tr>
<td>25</td>
<td>0.86 ± 0.090</td>
<td>1.58 ± 0.134</td>
</tr>
<tr>
<td>35</td>
<td>0.83 ± 0.085</td>
<td>1.01 ± 0.096</td>
</tr>
</tbody>
</table>

acclimation when assays were performed at 41 °C. At acclimation incubation temperatures, however, significant (P < 0.01) increases were observed, and again these represented a rise of about 300% between 5 and 35 °C. Q₁₀ values and thermal coefficients were similar to those of ‘membrane’ preparations, with the greatest increases in relative activity taking place in the mid-range region, i.e. 15-25 °C. Activity in relation to maximum activity followed much the same pattern as that for ‘membrane’ preparations. ‘Cytosol’ specific activities were almost identical to those previously reported for rainbow trout (Smeda & Houston, 1979). ‘Membrane’ activities were, however, lower than the values recorded for trout at comparable temperatures, and appeared to differ in thermosensitivity.

Activity per unit volume of cells

Because of changes in protein content with temperature, variations in specific activity do not necessarily indicate functional changes in carbonic anhydrase activity accompanying acclimation. Activity per unit volume of erythrocytes was therefore calculated (Table 2). When expressed in this way ‘cytosol’ activity assumes greater importance, accounting for approximately two-thirds of overall activity. Under acclimation temperature incubation conditions both ‘membrane’ and ‘cytosol’ activities increased in near-linear, and highly significant (P < 0.01) fashion between 5 and 25 °C. No further significant change was seen between 25 and 35 °C. By contrast, ‘membrane’ assays conducted at 41 °C provided no evidence of any consistent trend related to acclimation status. ‘Cytosol’ activities were somewhat higher at intermediate temperatures (15°, 25 °C) than at the extremes (5°, 35 °C). Activity as a proportion of maximum activity presented much the same picture as that encountered in relation to specific activity. Finally, in contrast to the situation in rainbow trout (Smeda & Houston, 1979) ‘cytosol’ activity consistently exceeded ‘membrane’ activity, and increased more than did the latter with acclimation to higher temperatures.

Since haematocrit also varied with temperature blood carbonic anhydrase activity was estimated on the basis of haematocrit values and activities per unit volume of cells (Fig. 2). Little variation was apparent in assays carried out at 41 °C. Increases in activity under the actual temperature conditions of the animals were, however, highly significant (P < 0.01).
DISCUSSION

The occurrence of distinct 'membrane' and 'cytosol' carbonic anhydrase activities of differing thermal sensitivity is compatible with the recent demonstration of carbonic anhydrase isoenzymes in eel, *Anguilla anguilla*, and flounder, *Platichthys flesus* (Girard & Istin, 1975; Carter, Auton & Dando, 1976). Although obvious distinctions in specific activity were apparent, the crude nature of these preparations precludes any suggestion that they are functionally analogous to the low- and high-activity CA I and CA II carbonic anhydrase isoenzymes typical of mammals.

Several features of change in carbonic anhydrase activity with temperature were of interest. In several instances activity, as measured at 41 °C, presented a very different pattern of variation with acclimation temperature than was the case when preparations were incubated at temperatures corresponding to those at which the system actually operates in the animal. This has also been reported for (Na/K)-ATPase systems (EC 3.6.1.3; ATPase) in several teleosts (Smith, 1970; Smith & Ellory, 1971; McCarty & Houston, 1977) and the carbonic anhydrases of rainbow trout (Houston & McCarty, 1978). Whatever the basis of these differences may be, they emphasize the interpretive pitfalls associated with the definition of enzyme activities at other than physiologically relevant temperatures.

From another viewpoint, goldfish and carp, like trout, are able to conserve plasma sodium and chloride levels over relatively broad temperature ranges despite significant changes in urinary, and presumably branchial depletion rates (Houston, Madden & DeWilde, 1970; Houston, 1973; Mackay, 1974; Murphy & Houston, 1974). Because
of the extreme temperature sensitivity of (Na/K)-ATPase (Giles & Vanstone, 1976; Russell & Chambers, 1976) it has been hypothesized that freshwater teleosts use the relatively thermostable gill and kidney carbonic anhydrase systems to establish base rates of sodium and chloride absorption and reabsorption by means of NH$_4^+$ and H$_3^+$:Na$^+$ and HCO$_3^-$:Cl$^-$ exchange mechanisms (McCarty & Houston, 1977). Consistent with this, the carbonic anhydrase activities of these tissues vary little with temperature in either the stenothermal trout or the eurythermal goldfish (Houston & McCarty, 1978; A. H. Houston & K. M. Mearow, unpublished observations). However, maintenance of sodium and chloride levels requires some means of amplifying net uptake of these ions as temperature rises. Thermal enhancement of branchial, and to a lesser degree, renal (Na/K)-ATPase activities in rainbow trout can be correlated with stabilization of plasma sodium (McCarty & Houston, 1977). In view of the Kerstetter-Kirschner (1972) hypothesis concerning chloride recruitment in this species, temperature-related increases in blood carbonic activity would be expected, and have been observed (Houston & McCarty, 1978; Smeda & Houston, 1979). The present study suggests that the more temperature-tolerant goldfish responds in much the same way, and this has been confirmed by evaluation of gill and kidney (Na/K)-ATPase and carbonic anhydrase activities in specimens acclimated to temperatures ranging from 5 to 35 °C (A. H. Houston & K. M. Mearow, unpublished observations). Additional support for this supposition can be seen in relationships between whole blood carbonic anhydrase activity – presumably the most appropriate measure of ability to drive HCO$_3^-$:Cl$^-$ exchange-specimen weight, and acclimation temperature. Oxygen consumption and gill area per unit body weight decrease as power functions of weight, while mean diffusion path length increases (Hughes, 1972; Hughes & Morgan, 1973). Thus, unless compensatory changes in lamellar permeability occur, the osmo- and ion-regulatory stresses (endosmosis, branchial and urinary electrolyte loss) faced by smaller specimens must be greater than those of larger individuals at the same temperature. Under such circumstances, if the Kerstetter-Kirschner hypothesis is valid, one would expect to see increases in blood carbonic anhydrase, but proportionally larger increases in smaller than in larger specimens. This is, indeed, the case. Whole blood carbonic anhydrase activity and weight are significantly correlated, and best-fitted by exponential functions, in which increases in temperature are accompanied by increases in both slope and intercept, i.e.

\[
5\, ^\circ\text{C}\text{ }\text{activity}\,(\mu\text{M}\text{ min}^{-1}\text{ ml}^{-1},\text{ whole blood}) = 0.856e^{-0.0643\text{ weight (g)}}
\]
\( (r = 0.836, P < 0.01) \)

\[
35\, ^\circ\text{C}\text{ }\text{activity}\,(\mu\text{M}\text{ min}^{-1}\text{ ml}^{-1},\text{ whole blood}) = 2.390e^{-0.9793\text{ weight (g)}}
\]
\( (r = 0.848, P < 0.01) \).

Variations in carbonic anhydrase activity are also consistent with the changes in red cell chloride content which have been observed in this species (Catlett & Millich, 1976) and the related carp (Houston & Smeda, 1979). Since chloride is a potent negative modulator of haemoglobin-oxygen affinity (de Bruin et al. 1974; Rollema et al. 1975) satisfaction of temperature-induced increases in oxygen demand may be associated, in part at least, with the changes in erythrocytic carbonic anhydrase activity accompanying acclimation.
Carbonic anhydrase activity of goldfish

Finally, osmoregulatory involvements in the heat death of freshwater fishes have frequently been suggested (e.g. Doudoroff, 1945; Brett, 1952), and are supported by studies on the effects of varying salinity on the thermal tolerances of euryhaline species (Garside & Jordan, 1968; Jordan & Garside, 1972). Precision of ionic regulation sometimes declines at high temperature and, in a few instances, marked deviations in plasma electrolyte levels are seen as temperature approaches near-lethal levels (Houston, 1973). In rainbow trout maximum activities of (Na/K)-ATPase and carbonic anhydrase (defined at 25 °C), and those resulting from incubations at acclimation temperatures are near-linear functions of temperature and intersect a value close to the upper incipient lethal temperature of this species. This suggests that heat death is associated with temperature conditions under which ion transport capacity is exceeded. Much the same is true of red cell carbonic anhydrase activity in the goldfish. Various estimates of activity extrapolate to 43.8 ± 1.57 °C, a temperature close to the ultimate upper lethal for this species (Fry, Brett & Clawson, 1942), and this further supports the view that heat death may, under some circumstances, be related to regulatory capacity.

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