Functional Significance of Isoenzymes in Thermal Acclimatization

ACETYLCHOLINESTERASE FROM TROUT BRAIN

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1. The effects of acclimatization temperature on the catalytic properties of acetyl-cholinesterase from rainbow-trout brain were examined. 2. Trout brain acetyl-cholinesterase occurs in two distinct forms. A single 'warm' variant of the enzyme is present after acclimatization to 17°C; a single 'cold' variant appears after acclimatization to 2°C. Both forms are present in fish after acclimatization to an intermediate temperature. 3. The K_m values of the enzyme variants for acetyl-choline are temperature-dependent, the lowest values coinciding with the acclimatization temperature at which each enzyme was induced. 4. It is concluded that the K_m–temperature relationship is adaptive, and that the critical process during thermal acclimatization, in cases where enzymes show sharp changes in K_m with temperature, is the synthesis of a new enzyme variant that is better suited for catalysis and control of catalysis under the conditions of the acclimatized state.

A number of studies have indicated the importance of changes in the central nervous system of fish during thermal acclimatization and the possible limiting role of the central nervous system in the overall acclimatization process. Prosser and co-workers (Roots & Prosser, 1962; Prosser & Farhi, 1965) have shown that the central nervous system of fish is more sensitive to temperature change than is the peripheral nervous system and, further, that the temperature for cold-block of a conditioned response in goldfish is affected by the temperature at which the response was established. Konishi & Hickman (1964) found that the midbrain response to electrical stimulation of the retina in rainbow trout kept at different temperatures showed compensatory changes in both nerve conduction velocity and central response times over an acclimatization period of several weeks.

The molecular mechanisms underlying these effects are not known (Baslow, 1967). In other systems, we have found that decreasing temperatures alter enzyme–substrate affinities in a manner analogous to that of positive modulators. With several enzymes this effect is large enough to compensate completely for decreased thermal energy (Behrisch, 1969; Hochachka & Somero, 1968). In view of the important role of AChE* in neural transmission (Nachmansohn, 1968) it was decided to examine temperature relationships of brain AChE from the eurythermal rainbow trout (*Salmo gairdnerii*).

* Abbreviation: AChE, acetylcholinesterase (acetyl-choline acetylhydrolase, EC 3.1.1.7).

MATERIALS AND METHODS

Experimental animals. Adult rainbow trout weighing approx. 200g were kept at 2, 12 or 17°C for at least 4 weeks. This species experiences annual temperature fluctuations between 2 and 18°C in many Western Canadian waters.

Preparation of AChE. The extraction was carried out at 4°C. Brains from 30–80 fish were homogenized in a small volume of water, freeze-dried and extracted twice with butan-1-ol and once with acetone to solubilize the enzyme. The acetone-dried powder was taken up in 10mM-tris–HCl buffer, pH7.2, and centrifuged at 30000g for 1h to remove insoluble material. The enzyme was further purified by $(NH_4)_2SO_4$ fractionation between 20 and 50% saturation. The final extract could be stored frozen in 5%-satd. $(NH_4)_2SO_4$ for at least 6 months without loss of activity. This preparation was dialysed against 10mM- tris–HCl buffer, pH7.2, at 4°C before use. For the determination of total AChE activity, brains were homogenized in ice-cold 10mM-tris–HCl buffer, pH7.2, at a concentration of 100mg of brain/ml.

Assay of AChE activity. AChE activity was assayed with an automatic titrator (Radiometer, Copenhagen, Denmark) operated as a pH-stat with 10mM-NaOH as titrant. The basic reaction mixture contained 10mM-tris–HCl buffer, pH7.2, at the assay temperature, enzyme and substrate, in a total volume of 2.0ml. Temperature was accurately controlled with a circulating water bath (Lauda Brinkman, K-2/R) coupled to a water jacket surrounding the reaction vessel.

Electrophoresis. AChE preparations were examined by standard polyacrylamide-gel electrophoresis (Davis, 1964) at 4°C, with a 4% stacking gel (prepared in 62mM-tris–HCl buffer, pH6.2), 7% separating gel (prepared in

380mM-tris–HCl buffer, pH8.9), and tris–glycine tank buffer, pH8.9 (5mM-tris, 30mM-glycine). Gels were run for 90min at 3mA/tube and stained for esterase activity with α-naphthyl acetate (40mg/100ml) and Fast Blue RR salt (70mg/100ml) in 40mM-tris–HCl buffer, pH7.1 (Allen, Popp & Moore, 1965).

RESULTS AND DISCUSSION

Multiple forms of trout brain AChE. At least seven bands of esterase activity were observed in trout brain extracts. Specific AChE bands were detected by inhibition with 0.1mM-eserine and with 10μM-284C51 [dimethobromide of 1,5-di-(N-allyl-N-methyl-p-aminophenyl)pentan-3-one; Burroughs Wellcome, Beckenham, Kent, U.K.]. AChE bands obtained with preparations from fish acclimatized to 2, 12 and 17°C are shown in Fig. 1.

AChE from cold-adapted trout shows a distinctly slower migration rate than does the enzyme from warm-adapted fish; trout acclimatized to 12°C possess both enzyme types. These results were verified by subjecting mixtures of the brain extracts to polyacrylamide-gel electrophoresis.

Characterization of trout brain AChE enzymes. AChE enzymes have been defined by Augustinsson (1957) as eserine-sensitive esterases that are inhibited by high acetylcholine concentrations (generally 3–5mM) and that split acetylcholine at a much higher rate than butyrylcholine. The compound 284C51 at concentrations of 1–10μM gives about 100000-fold greater inhibition of AChE than of other cholinesterases (Austin & Berry, 1953).

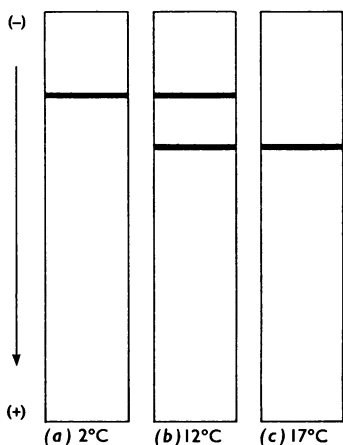Substrate-saturation plots for brain extracts from trout acclimatized to 2 and 17°C are shown in

Fig. 1. AChE bands obtained by disc electrophoresis of brain extracts from trout acclimatized to 2°C (a), 12°C (b) and 17°C (c).
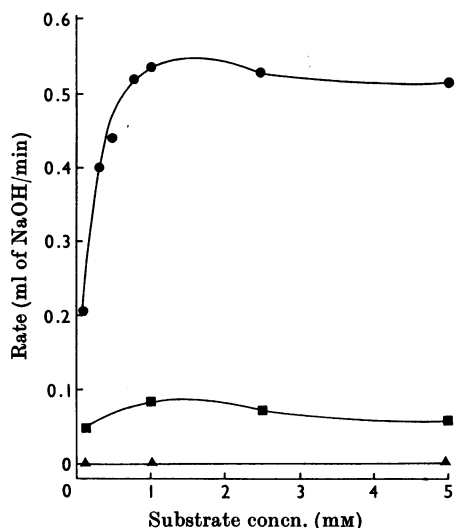

Fig. 2. Substrate specificity of AChE from 2°C-acclimatized trout. ●, Acetylcholine chloride; ■, propionylcholine chloride; ▲, butyrylcholine chloride. The assay temperature was 2°C.
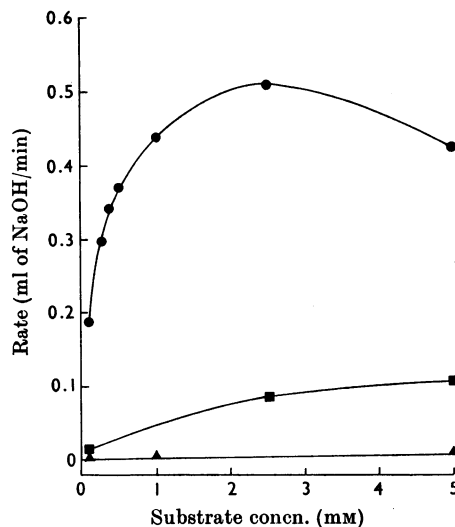

Fig. 3. Substrate specificity of AChE from 17°C-acclimatized trout. ●, Acetylcholine chloride; ■, propionylcholine chloride; ▲, butyrylcholine chloride. The assay temperature was 17°C.

Figs. 2 and 3. Both preparations show greatest activity with acetycholine and in each case substrate inhibition occurs at concentrations above about 3mM-acetylcholine. Hydrolysis of mM-acetylcholine was completely inhibited by both 50μM-eserine and μM-284C51. The bell-shaped pH–activity curves (Fig. 4) are similar to those obtained
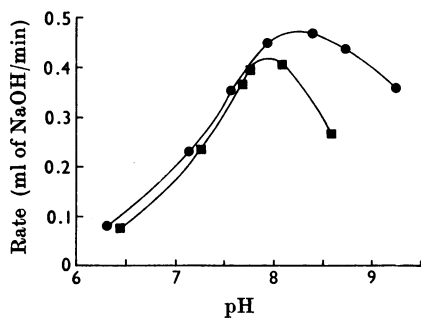
Fig. 4. pH–activity plot for trout brain AChE enzymes. ●, 17°C-acclimatized trout (assay temperature 17°C); ■, 2°C-acclimatized trout (assay temperature 2°C). 10mM-Sodium phosphate buffer was used in the range pH 6–8 and 10mM-tris–HCl buffer containing 10mM-NaCl in the range pH 7.5–9.5.
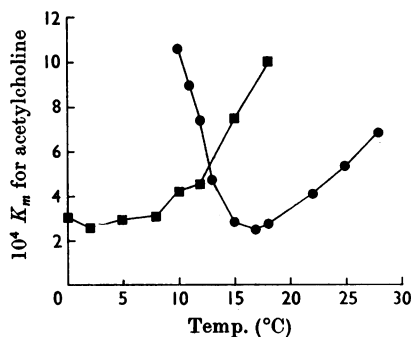

Fig. 6. Effect of assay temperature on K_m of AChE for acetylcholine from trout acclimatized to 17°C (●) and to 2°C (■). AChE was assayed at acetylcholine concns. in the range 0.1–5mM and K_m values were determined from double-reciprocal plots ($1/v$ versus $1/$[acetylcholine]).
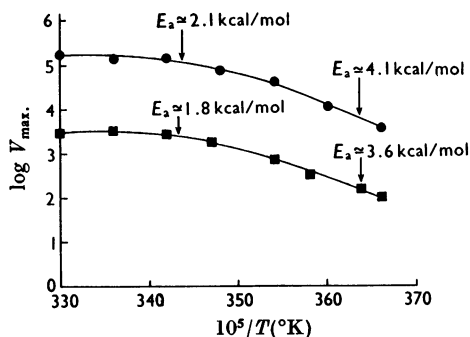

Fig. 5. Arrhenius plot of AChE activity ($\log V_{max.}$ versus $1/T°$K) for 17°C-acclimatized trout (●) and 2°C-acclimatized trout (■).

with AChE enzymes from a variety of sources (Bernsohn, Barron & Hedrick, 1963; Bull & Lindquist, 1968; Silman & Karlin, 1967). These results indicate that essentially all of the esterase activity of trout brain extracts detected by the assay can be attributed to AChE.

Effect of temperature on maximum velocity. Arrhenius plots of $\log V_{max.}$ versus $1/T$ for AChE enzymes from warm-adapted and cold-adapted trout are shown in Fig. 5. In each case $V_{max.}$ of the reaction increases with temperature over the thermal range of the trout. It has been argued that rates of enzyme activity in cold-adapted organisms may be maintained through a lowering of activation energy (Vroman & Brown, 1963). For the trout AChE enzymes the curved Arrhenius plots yield energies of activation (E_a) that decrease as the temperature is raised. Similarly curved plots have been reported for electric-eel AChE. Wilson &

Cabib (1956) interpreted the decrease in activation energy with temperature for electric-eel AChE in terms of a change in the rate-limiting step for the overall reaction, and it has also been suggested that plots of $\log V_{max.}$ versus $1/T$ need not give straight lines when both $V_{max.}$ and K_m vary with temperature. Non-linear Arrhenius plots obtained with amino acid oxidase have been related to a temperature-dependent transition of the enzyme between two conformations (Koster & Veeger, 1968; Massey, Curti & Ganther, 1966). Evidence has been presented for similar temperature-dependent transitions between multiple forms of serum cholinesterase and AChE from erythrocytes (Main, 1969).

Activation energies obtained by drawing tangents to the curves at 2 and 17°C for AChE enzymes from 'warm'- and 'cold'-adapted trout are given in Fig. 5. The values for the two enzymes are not significantly different.

Effect of temperature on K_m. The relationship between K_m for acetylcholine and assay temperature for the brain preparations from trout acclimatized to 2 and 17°C are shown in Fig. 6. It is apparent that, over the upper part of the biological thermal range, the affinities of the two trout enzymes for acetylcholine vary with temperature and approach maximal values (minimum K_m) at temperatures corresponding to those at which the fish were acclimatized. Similar relationships between habitat temperature and K_m have been demonstrated for lactate dehydrogenases (Hochachka & Somero, 1968), pyruvate kinases (Somero & Hochachka, 1968), glucose 6-phosphate dehydrogenases and 6-phosphogluconate dehydrogenases (G. N. Somero, personal communication), fructose diphosphatases (Behrisch, 1969) and choline acetyltransferases (Hebb, Morris & Smith, 1969) from several species

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of fish and crustaceans inhabiting different thermal environments.

At temperatures above that at which K_m is at a minimum the physiological significance of the K_m-temperature relationship is clear. Although a rise in temperature would be expected to increase the velocity of the enzymic reaction, this effect is neutralized by a decrease in enzyme–substrate affinity. Hence the overall reaction rate remains relatively independent of temperature. This type of temperature-independence is characteristic of both forms of trout AChE. The 'warm' form shows this relationship at temperatures above about 17°C; the 'cold' form shows the relationship above about 2°C. This effect is reflected in the rates of acetylcholine hydrolysis at substrate concentrations approaching the minimum K_m (Table 1). An estimate of 0.216 mM for the concentration of acetylcholine released into the synaptic space of vertebrate motor end-plates (Namba & Grob, 1968) corresponds closely to the minimum K_m values of both forms of trout AChE.

At lower thermal extremes the K_m–temperature relationship is reversed. For the 'warm' form of the enzyme the K_m rises sharply as the temperature falls below 15°C, reaching an extrapolated K_m at 8°C that is about 10 times the minimum value. Thus at 2°C the K_m of the enzyme would be so high as to make the enzyme essentially inactive at low and presumably physiological substrate concentrations.

A feature of the two forms of trout AChE is that the minimum values of the K_m are similar, although they occur at very different temperatures. Thus at K_m concentrations of substrate, the reaction catalysed by the 'cold' enzymes at 2°C will proceed at a lower rate than the reaction catalysed by an equal amount of the 'warm' enzyme at 17°C. There are a number of factors that could conceivably act to raise the rate of acetylcholine hydrolysis in the cold acclimatization state. Hickman, McNabb, Nelson, Van Breeman & Comfort (1964) observed rapid changes in brain Na^+ and K^+ contents, and longer-term changes in brain Cl^- contents in rainbow trout transferred from 16 to 6°C. Preliminary studies of the effect of ions on AChE from 2°C-adapted trout (Table 2) show that increasing ionic strength leads to a marked increase in both K_m and $V_{max.}$ of acetylcholine hydrolysis. However, at lower acetylcholine concentrations the rate of the reaction generally decreases as ionic strength is increased.

An increase in intracellular and blood pH of about 0.014 pH units/°C when the environmental temperature is lowered has been observed in several poikilotherms (Rahn, 1965; Reeves & Wilson, 1969). With trout AChE, a fall in temperature from 17 to 2°C could result in a 12% increase in the rate of acetylcholine hydrolysis, if the pH–activity relationship (Fig. 4) holds at physiological substrate concentrations. Another possibility is that rate compensation is achieved through an increase in the

Table 1. *Effect of temperature upon rates of acetyl-choline hydrolysis at K_m concentrations of substrate (0.25 mM) for trout AChE enzymes*

The standard assay system in 10 mM-tris–HCl buffer, pH 7.2, was used.

Enzyme	Temperature at minimum K_m (°C)	Assay temperature (°C)	Rate of hydrolysis at assay temp./rate at minimum K_m temp.
2°C-AChE	2	0	0.80
		2	1.00
		5	1.10
		8	1.10
		12	1.20
17°C-AChE	17	11	0.61
		13	0.80
		17	1.00
		22	1.00
		27	1.20

Table 2. *Effect of salts on K_m and reaction velocity for hydrolysis of acetylcholine by AChE from 2°C-acclimatized trout*

The standard assay in 10 mM-tris–HCl, pH 7.2 (control) was carried out. The assay temperature was 2°C.

Salt added (final concn.)	$10^4 \times K_m$ (M)	Concn. of acetylcholine ...	Relative AChE activity		
			25 mM	50 mM	Saturating
mM-NaCl	3.3		0.36	0.58	0.96
5 mM-NaCl	4.1		0.35	0.58	1.06
10 mM-NaCl	7.7		0.31	0.61	1.50
10 mM-KCl	4.9		0.35	0.61	1.20
2 mM-MgCl$_2$	4.4		0.45	0.70	1.33
5 mM-MgCl$_2$	7.0		0.32	0.62	1.42
Control	2.7		0.44	0.63	1.00

Table 3. *Specific activity of brain AChE from trout acclimatized at 2°C and at 17°C for 35 days*

Whole brain homogenates (100mg of brain/ml) were assayed in 10mм-tris–HCl buffer, pH 7.2, with mм-acetylcholine as substrate. Specific activities are expressed as μmol of acetylcholine hydrolysed/h per mg of protein. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Acclimatization temperature (°C)	No. of brains assayed	Assay temp. ...	AChE specific activity		
			18°C	10°C	2°C
2	12		10.1 ± 0.1	8.6 ± 0.3	6.2 ± 0.3
17	11		10.1 ± 0.3	8.4 ± 0.1	6.2 ± 0.1

total amount of enzyme present in the cold, as proposed by Baslow & Nigrelli (1964) to account for compensation in brain cholinesterase activity in thermally acclimatized killifish. However, measurement of specific activity of AChE from trout acclimatized to 2 and 17°C for 35 days (Table 3) failed to show any significant difference in this species.

The temperature-dependent production of isoenzymes displaying K_m–temperature relationships similar to those of the trout brain AChE enzymes has been observed with salmonid lactate dehydrogenases (Hochachka & Somero, 1968) and pyruvate kinases (G. N. Somero, personal communication.) In all cases, the functional advantage of employing 'better' enzymes (as opposed to producing altered quantities of a single enzyme species) is not a decrease in Q_{10}, for complete rate compensation would demand that the 'cold' isoenzymes have drastically lower K_m values than the 'warm' isoenzymes. Rather, the primary function of the acclimatization process is the production of enzymes with K_m values in a range likely to be optimum for regulation of catalytic activity. Thus small changes in substrate concentration or small change in K_m can lead to large changes in the activities of 'cold', but not of 'warm', forms of these enzymes at low temperatures. In evolutionary terms, it appears that there is a strong selection for enzymes permitting large changes in activity in response to physiological changes in substrate concentrations. This is reflected in the patterns of enzyme variants produced during acclimatization and those selected during evolutionary adaptation.

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