Temperature and Pressure Adaptation of the Binding Site of Acetylcholinesterase

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1. Studies with a carbon substrate analogue, 3,3-dimethylbutyl acetate, indicate that the hydrophobic contribution to binding at the anionic site of acetylcholinesterase is strongly disrupted at low temperatures and high pressures. 2. Animals living in different physical environments circumvent this problem by adjusting the enthalpic and entropic contributions to binding. 3. An extreme example of this adaptational strategy is supplied by brain acetylcholinesterase extracted from an abyssal fish living at 2°C and up to several hundred atmospheres of pressure. This acetylcholinesterase appears to have a smaller hydrophobic binding region in the anionic site, playing a measurably decreased role in ligand binding.

Acetylcholinesterase (EC 3.1.1.7), which catalyses the reaction:

of any acetylcholinesterase and the positive quaternary ammonium ion would presumably be stabilized

is ubiquitously distributed in the nervous tissue of animals. The enzyme has been intensively studied because of its fundamental role in neural function (Nachmansohn, 1971) and because of the large potential for practical use implicit in any modification of its activity. As a result, it has been clear for some time now that the active site consists of two subsites, an anionic site and an esteratic site. The first is primarily involved in binding, whereas the second is the true catalytic site, the site at which hydrolysis occurs. Both coulombic forces, involving the positively charged quaternary ammonium head of the substrate, and hydrophobic interactions, between the anionic site and the methyl groups on the nitrogen atom, contribute to binding [see Froede & Wilson (1972) for a review].

Although it is believed that hydrophobic interactions contribute a greater fraction to the overall free energy of binding, I reasoned that the quantitative importance of the two binding contributions would vary in different species according to the temperature and pressure at which the enzyme functions. My reasoning was based on the observation that both binding contributions derived from non-covalent or 'weak' chemical bonds, which are highly sensitive to temperature and pressure. For example, the electrostatic interactions between the anionic site

and strengthened at low temperatures, whereas the hydrophobic interactions would probably be weakened. If that were the case, we might expect enzyme-substrate affinity to show important temperature and species dependence. Earlier investigations of brain acetylcholinesterase from temperate-zone fishes (Baldwin & Hochachka, 1970) and from polar and tropical fishes (Baldwin, 1971) in fact indicate a complex K_m -temperature relationship for each enzyme examined. My present study attempts to sort out the effect of temperature and pressure on both binding contributions by utilizing substrate analogues and 'anionic site-specific' inhibitors.

Materials and Methods

Enzyme preparation

Four enzyme preparations, obtained from a mammal (ox), a stenothermal tropical fish (electric eel), a eurythermal tropical fish (dolphin fish), and a highly cold-adapted abyssal fish (*Antimora rostrata*), were used in these studies as a routine. Purified type III acetylcholinesterase from *Electrophorus electricus* was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A., and after dialysis was used in kinetic experiments without further purification. Its specific

activity was about 900 μmol of product/min per mg of protein at 37°C, with the assay described below. The other three enzymes were extracted from brain tissue of freshly killed animals.

Fresh ox brain, obtained from a local slaughter house, was chopped up, then homogenized in a Sorvall Omnimixer at top speeds for a period of about 2 min. The homogenate (1:3, w/v) was centrifuged at low speeds to remove debris. Acetylcholinesterase in the supernatant was extracted and solubilized in 50 mM Tris-HCl buffer (pH 7.5)-100 mM-KCl-1% Triton X-100 for about 60 min (McIntosh & Plummer, 1973). After dialysis, the supernatant was applied to an equilibrated Sephadex G-200 column (75 cm $\times$ 2.5 cm) and eluted with Tris-HCl buffer. Fractions were collected with an LKB fraction collector, and those containing highest acetylcholinesterase activity (about 5 μmol of product/min per mg of protein), corresponding to peak C of Chan *et al.* (1972), were pooled and further dialysed before being used for kinetic experiments.

Dolphin fish (*Coryphaena hippurus*) were caught by line and baited hook in surface waters (26°C) off the Kona coast of Hawaii. The brain, which in this species is relatively large, was quickly excised, placed in ice-cold buffer, homogenized, and prepared as described above except that in this case the Sephadex G-200 step was omitted because of a much smaller amount of material. Enzyme activity in the initial extract was about 80 μmol of product formed/min per g wet wt. of nervous tissue, and the final preparation represented about a 10-fold purification of the enzyme.

For comparative purposes I also captured an abyssal fish, *Antimora rostrata*, using a free-vehicle capture technique previously described (Phleger & Soutar, 1971). All of my successful vehicles were dropped in about 1100 fathoms of water off the Kona coast of Hawaii. At this depth, the water temperature is 2°C and the hydrostatic pressure is about 20.5 MPa (205 atm). To reproduce these physical conditions in certain kinetic experiments, I used a temperature-controlled high-pressure cell built into an SP.1800 Unicam recording spectrophotometer (Mustafa *et al.*, 1971). Brain acetylcholinesterase from freshly captured *Antimora* was prepared by methods identical with those used for the dolphin fish. Enzyme activity in the initial extract was about 2 μmol /min per mg and the final preparation also represented about a 10-fold purification.

Enzyme assay

Most of my routine acetylcholinesterase assays used acetylthiocholine, obtained from Sigma Chemical Co., as the substrate. In this assay, the rate of thiocholine production is followed at 412 nm by its reaction with 5,5'-dithiobis-(2-nitrobenzoic acid) (0.2 mM in 50 mM-Tris-HCl, pH 7.5). Enzyme activity

was determined at various temperatures and pressures in this solution with various concentrations of substrate, and in some cases various concentrations of inhibitors. All my acetylcholinesterase preparations were fully inhibited by 0.1–1.0 μM -neostigmine. In experiments using 3,3-dimethylbutyl acetate as substrate, the rate of hydrolysis was followed spectrophotometrically at 620 nm by including a pH indicator, Bromothymol Blue ($pK = 7.0$), in a weakly buffered assay mixture as described by Changeux (1966). Michaelis constants (K_m values) for the enzymes under various conditions were calculated from double-reciprocal plots; all values reported are averages of two to four determinations and their range of error is $\pm 5\%$.

Calculation of thermodynamic parameters for enzyme-inhibitor dissociation

In a number of experiments, it was important to determine the ΔG° , ΔH° and ΔS° for binding of specific inhibitors competing with the substrate for the anionic site. The K_i in each case was determined by plotting $1/v$ against inhibitor concentration at minimally two substrate concentrations, the experimental error being in the same range as for the K_m calculations. For enzyme-inhibitor dissociation the values of ΔH° were calculated from the slopes of straight-line plots of $\ln K_i$ against $1/T$. The free-energy change in enzyme-inhibitor dissociation was calculated from the relationship:

$$\Delta G^\circ = -RT \ln K_i$$

The ΔS° changes were calculated on the basis of the values of ΔG° and ΔH° :

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T$$

It is important to emphasize that calculation of these parameters depends on K_i estimates and that the percentage error in estimating the thermodynamic parameters increases. As a first approximation, I consider this a minor difficulty since in comparing different enzymes it is the direction of change rather than the absolute magnitude of change that is of major interest.

Results and Discussion

Effect of temperature and pressure on maximum velocity

The effect of temperature on eel enzyme activity under optimal concentrations of acetylthiocholine is almost identical with that observed with the true substrate (Baldwin & Hochachka, 1970). Arrhenius plots with both substrates are curvilinear with Q_{10} values of about 1.3 and activation energies of 10.5 kJ/mol (2.5 kcal/mol), calculated between 15° and 25°C. With a carbon analogue, 3,3-dimethylbutyl acetate,

Fig. 1. Plot of the Michaelis constant (K_m) against temperature for acetylthiocholine, $\text{Me}_3\text{N}^+-\text{CH}_2-\text{CH}_2-\text{S}-\text{Ac}$, and for the uncharged substrate 3,3-dimethylbutyl acetate, $\text{Me}_3\text{C}-\text{CH}_2-\text{CH}_2-\text{O}-\text{Ac}$

In each case, the K_m was determined by reciprocal plots ($1/v$ versus $1/s$). The animal used was the electric eel. Δ, Carbon analogue; ○, acetylthiocholine.

as the substrate, the Q_{10} is about 2 and the Arrhenius activation energy is about 50 kJ/mol (12 kcal/mol).

With optimal concentrations of substrate, the electric-eel enzyme is inhibited by pressure at both low (6°C) and at high (25°C) temperatures. At 6°C, the volume change of activation is 52 cm^3/mol , calculated from the following equation:

$$\Delta V^\ddagger = 2.3 RT \frac{\log k_{p_1} - \log k_{p_2}}{p_2 - p_1}$$

where the gas constant (R) has the value of 82.07 cm^3/mol , k_{p_1} and k_{p_2} are the rate constants at pressures p_1 and p_2 atmospheres, T is the temperature in degrees Kelvin, and $\Delta V^\ddagger$ is the volume change of activation. With the carbon analogue as substrate, the reaction velocity of the eel enzyme under optimal conditions is pressure independent to about 40 MPa (400 atm), but is increased as pressure is further raised. Between 0.1 and 20 MPa (1 and 200 atm) the $\Delta V^\ddagger$ is therefore essentially zero, whereas between 40 and 80 MPa (400 and 800 atm), $\Delta V^\ddagger$ is about $-51 \text{ cm}^3/\text{mol}$.

Effect of temperature on the K_m for a carbon substrate analogue

With purified acetylcholinesterase from the eel, it was important at the outset to reconfirm the effect

Fig. 2. Plot of the K_i for dimethylammonium ion against temperature for electric-eel acetylcholinesterase

The K_i at each temperature was determined by Dixon plots ($1/v$ versus inhibitor concentration) with at least two different substrate concentrations.

of temperature on the apparent enzyme-substrate affinity. Under our assay conditions, the K_m for acetylthiocholine does not change much between about 10° and 35°C; however, below 10°C, the K_m shows a strongly inverse temperature dependence, increasing about fivefold at 2°C (Fig. 1). Both the absolute values of the K_m and its temperature dependence are in essential agreement with previous studies utilizing acetylcholine, the true substrate for the reaction (Baldwin & Hochachka, 1970; Baldwin, 1971). I believe that in both cases the decrease in enzyme-substrate affinity at low temperature is primarily caused by a decreased hydrophobic contribution to binding. Support for this concept comes from a comparable experiment in which the carbon analogue, 3,3-dimethylbutyl acetate, is used as a substrate. This compound is very similar in shape and size to the true substrate but differs, of course, in being uncharged. Binding at the anionic site presumably is determined largely by hydrophobic interactions.

Under my conditions, at high (35°C) temperatures, the apparent K_m for the carbon analogue is about 10-fold higher than that for acetylthiocholine. As temperature falls, there is a dramatic increase in the K_m , and, by 2°C, it is 11-fold higher than at 35°C (Fig. 1). At low temperatures the difference between the charged and uncharged substrate is also greatly accentuated, the K_m for the carbon analogue being over 20 times greater than the K_m for acetylthiocholine. If the K_m for the carbon analogue is a valid estimate of the dissociation constant, an assumption not yet experimentally verified, binding proceeds with a ΔG° of -4.2 kJ/mol (-3.4 kcal/mol), ΔH° of 54 kJ/mol (12.8 kcal/mol) and ΔS° of $227 \text{ J/mol per } ^\circ\text{K}$ ($54.3 \text{ cal/mol per } ^\circ\text{K}$).

Table 1. Effect of temperature on the K_m for acetylthiocholine calculated from plots of $1/v$ against $1/s$

Acetylcholinesterases were extracted from brain of organisms living at widely different body temperatures.

Biological temperature (°C)	Species	K_m (mM) at:			
		6°C, 101 kPa	6°C, 69 MPa	26°C, 101 kPa	26°C, 69 MPa
37	Ox	0.6		0.2	
15–30	Dolphin fish	0.05	0.07	0.1	0.1
2	<i>Antimora</i>	0.08	0.03	0.1	0.08

Fig. 3. Plot of the Michaelis constant for the carbon analogue substrate, 3,3-dimethylbutylacetate, and the K_i for dimethylammonium chloride, against pressure

Enzyme source was electric eel. Temperature, 60°C. Other conditions were as described in the Materials and Methods section. Δ , Carbon analogue; $\bullet$, dimethylammonium ion.

These effects of temperature on the enzyme-carbon analogue interactions are opposite in sign to the effects of temperature on the coulombic contribution to binding. Some indication of the latter can be obtained from studies with inhibitors that compete with the substrate for binding at the anionic site. For relatively simple inhibitor compounds such as dimethylammonium ion, binding is probably determined largely by electrostatic interactions between the quaternary ammonium ion and the anionic site on the enzyme (Froede & Wilson, 1972). Not surprisingly, there is a fairly direct linear relationship between the K_i for this compound and temperature (Fig. 2), presumably owing to the stabilizing effects of low temperature on electrostatic interactions.

K_m -temperature relationships for brain acetylcholinesterases in different species

The results in Figs. 1 and 2 emphasize the disrupting effects of low and high temperature, respectively, on the hydrophobic and coulombic contributions to binding. To gain some insight into how animals handle this problem I compared the effect of temperature on the K_m for acetylthiocholine for brain enzymes obtained from species whose thermal environment differs widely. Table 1 shows that the low-temperature behaviour of acetylcholinesterase depends critically on the animal's thermal environment. At 6°C, for example, bovine brain enzyme shows the lowest enzyme-substrate affinity (perhaps reflecting low-temperature disruption of hydrophobic binding contributions), whereas the highest enzyme-substrate affinity is observed for the *Antimora* enzyme at low temperature and high [69 MPa (10000 lb/in²)] pressures.

Since *Antimora* is an abyssal species normally living at 2–3°C and up to several hundred atmospheres of pressure, a full comparison of its brain acetylcholinesterase with that of the dolphin fish required studies at different pressures. It will be evident that the two enzymes differ markedly in their pressure response (Table 1). In the case of the *Antimora* enzyme, the K_m is decreased by pressure, whereas the enzyme from the dolphin fish shows an increase in the K_m with pressure. Since high pressure stabilizes some weak-bonding interactions but disrupts hydrophobic ones (Suzuki & Taniguchi, 1972), this result also indicates that the quantitative significance of the two (coulombic against hydrophobic) contributions to substrate binding by acetylcholinesterase is carefully tailored during evolutionary adaptation to the physical environment. Further evidence for this conclusion and a potential explanation of the observed differences between acetylcholinesterases from surface and abyssal species comes from studies of the effects of pressure on enzyme-ligand interactions.

Effects of pressure on K_m and K_i values

Fig. 3 shows that the K_m for the carbon analogue is remarkably pressure-sensitive. At 20 MPa (200 atm), the value increases fourfold; at 40 MPa (400 atm),

Table 2. *Thermodynamic parameters for the association of brain acetylcholinesterase-phenyltrimethyl ammonium complex*

These parameters were measured at 298°K.			
Enzyme source	ΔG^0 (kJ/mol)	ΔH^0 (kJ/mol)	ΔS^0 (J/mol per °K)
Ox brain	-25.2	-6.8	59.2
Dolphin-fish brain	-25.2	-1.7	80.2
<i>Antimora</i> brain	-25.2	-26.0	-2.1

it increases by nearly sevenfold. If our assumption is correct that binding of the carbon analogue is due largely to hydrophobic forces, the result demonstrates a dramatically disrupting effect of pressure on this contribution to binding.

In contrast, high pressure appears to favour non-hydrophobic contributions to binding. Thus the K_i for the simple inhibitor, dimethylammonium ion, decreases greatly with pressure (Fig. 3). At 20 MPa (200 atm), the K_i is about two-thirds of the value at 0.1 MPa (1 atm) and 60 MPa (600 atm) it is less than one-fifth of the control value.

From these results (Fig. 3), it appears that the differences noted between surface and abyssal species could be readily explained if the percentage hydrophobic contribution to binding were decreased in the enzyme from the abyssal species. We attempted to gain more evidence for this idea from additional studies with 'anionic-site-specific' inhibitors.

Temperature effects on enzyme-inhibitor interactions

From earlier studies it is well known that any substituted ammonium ion, especially a tertiary or a quaternary ammonium ion, is a potential inhibitor of acetylcholinesterase because it is capable of binding at the anionic site. Moreover, such studies emphasize that a significant portion of the anionic site must be hydrophobic, since large hydrocarbon chains or rings almost always increase binding [see Froede & Wilson (1972) for a review of this topic]. To assess further if the hydrophobic region of the anionic site is equally important in species living in different thermal and pressure regimes, we examined the thermodynamics of binding of a good 'hydrophobic' inhibitor, phenyltrimethylammonium ion. We found that the overall ΔG^0 of binding -25.2 kJ/mol, (-6.0 kcal/mol), is remarkably similar in all four enzymes examined, but the enthalpic and entropic contributions to the free-energy change in enzyme-inhibitor association vary dramatically with the physical environment of the organism. The data for the three brain enzymes are summarized in Table 2. In the case of the brain enzyme from both ox and the dolphin fish, enzyme-inhibitor association proceeds with large positive ΔS^0 changes. In contrast, in *Antimora*, which is adapted to extremely low temperatures and high pressures (neither of which favour hydrophobic interactions), the enzyme-inhibitor association proceeds

with essentially zero entropy change (Table 2). The simplest interpretation of this result is that the *Antimora* acetylcholinesterase has a 'smaller' hydrophobic pocket in the anionic site, playing a measurably diminished role in binding inhibitor, and by implication, in binding substrate. The decreased $T\Delta S^0$ contribution to the free energy of binding is precisely compensated by an increased enthalpic contribution (Table 2). In consequence, the overall ΔG^0 of binding is unchanged.

When less effective inhibitors are used, such as ethylammonium, triethylammonium, dimethylammonium or tetramethylammonium ions, some differences can be observed between these enzymes (Hochachka *et al.*, 1975). However, compared with phenyltrimethylammonium ion, the binding of these relatively poor inhibitors to the anionic site depends less on hydrophobic bonding. Hence, with these inhibitors, differences between the four enzymes examined are small and are difficult to interpret.

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References

- Baldwin, J. (1971) *Comp. Biochem. Physiol. B* **40**, 181–187
- Baldwin, J. & Hochachka, P. W. (1970) *Biochem. J.* **116**, 883–887
- Chan, S. L., Shirachi, D. Y., Bhargava, H. N., Gardner, E. & Trevor, A. J. (1972) *J. Neurochem.* **19**, 2747–2758
- Changeux, J. P. (1966) *Mol. Pharmacol.* **2**, 369–392
- Froede, H. C. & Wilson, I. B. (1972) *Enzymes* **5**, 89–114
- Hochachka, P. W., Storey, K. B. & Baldwin, J. (1975) *Comp. Biochem. Physiol.* in the press
- McIntosh, C. H. S. & Plummer, D. T. (1973) *Biochem. J.* **133**, 655–665
- Mustafa, T., Moon, T. W. & Hochachka, P. W. (1971) *Amer. Zool.* **11**, 451–466
- Nachmansohn, D. (1971) *Proc. Nat. Acad. Sci. U.S.A.* **68**, 3170–3174
- Phleger, C. F. & Soutar, A. (1971) *Amer. Zool.* **11**, 409–418
- Suzuki, K. & Taniguchi, Y. (1972) *Symp. Soc. Exp. Biol.* **26**, 103–124