Biochimica et Biophysica Acta, 525 (1978) 93—102

BBA 68490

TEMPERATURE AND pH EFFECTS WITH IMMOBILIZED ELECTRIC EEL ACETYLCHOLINESTERASE

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(Received January 24th, 1978)

Summary

Kinetic studies were made with 2 forms of immobilized acetylcholinesterase: enzyme trapped in polyacrylamide gel which was cut into slices; and enzyme attached to the inner surface of nylon tubing. Rates were measured at substrate concentrations which were low and high with reference to the Michaelis constant, and over the temperature range 16—40°C. Low activation energies (1.7—2.7 kcal mol^{-1}) were obtained at low substrate concentrations, indicating diffusion control. At high substrate concentrations the Arrhenius plots were non-linear and the activation energies substantially higher, and there is less diffusion control.

With enzyme-polyacrylamide slices, there was a continuous increase in rate with increasing pH, in contrast to the bell-shaped behavior with free enzyme. A theoretical treatment suggests that this is due to the lowering of local pH as a result of the acid released in the hydrolysis.

Introduction

The behaviour of an immobilized enzyme may differ from that in free solution as a result of the following factors [1—7]:

(a) The conformation of the enzyme may be altered in the immobilization.

(b) The substrate may not be equally partitioned between the support and the bulk solution.

(c) The interaction between an immobilized enzyme and its substrate takes place in a different microenvironment from that existing in free solution.

(d) There may be complete or partial diffusion control.

Previous work in this laboratory has been concerned with enzymes trapped in polyacrylamide gels [8,9] and with the flow kinetics of reactions catalyzed by enzymes attached to the inner surface of tubing [10—12]. The present paper is concerned with temperature and pH effects on the activity of electric

eel acetylcholinesterase immobilized in polyacrylamide gel and supported on the inner surface of nylon tubing.

Materials and Methods

Chemicals

Electric eel acetylcholinesterase (type III), acetylthiocholine, 5,5′-dithiobisnitrobenzoic acid, and glutaraldehyde were obtained from the Sigma Chemical Co., St. Louis, Mo. *N,N,N′,N′*-tetramethylene-ethylene diamine, *N,N′*-methylene bisacrylamide, acrylamide monomer and Fotoflow solution were obtained Eastman Organic Chemicals, Rochester, N.Y. The nylon tubing (internal diameter 0.1 cm) was obtained from John Tullis, Tullibody, Alloa, Scotland.

All solutions were made up in a buffer consisting of 0.15 M NaCl, 2 mM phthalate, 2 mM phosphate and 2 mM borate, adjusted with dilute NaOH solution.

Preparation of immobilized enzyme systems

Acetylcholinesterase trapped in a 15% polyacrylamide gel was prepared according to the method described by Ngo and Laidler [9]. Polymerization was carried in a Fotoflow-coated glass tube 1 cm in diameter. In typical entrapment experiments, 1.5 ml 60% acrylamide solution containing 5% bisacrylamide, 0.5 ml 2% tetramethylethylene diamine, 0.1 ml 2.8% ammonium persulfate, and 3.9 ml enzyme in buffer solution were mixed and stirred vigorously for 3 min and the whole mixture was poured into the glass tube with one end wrapped with parafilm. After gelation was complete, the gel was extruded from the glass tube, cut into 2-cm lengths, and placed on a Model-880 American Optical microtome where it was frozen in a stream of dry CO_2. The frozen gel was sliced into various thicknesses by manual adjustment of the blade-to-sample distance. After measurement of the thickness, the slices were stirred in a buffer solution of specified pH for 2 min for pH equilibration and for removal of substrate and product between rate measurements.

Immobilization of acetylcholinesterase on the inner surface of a nylon tube was done according to the method of Ngo and Laidler [11]. The inner surface of 1 m nylon tube was partially hydrolyzed by pumping 4.5 M HCl through the tube for 15 min af a flow rate of 5 ml min^{-1} at room temperature. Hydrolysis was stopped by pumping distilled water through the tube for 15 min at 5 ml min^{-1}. The tube was then rinsed with 500 ml 0.2 M sodium bicarbonate buffer (pH 9). Following the bicarbonate treatment, the nylon tube was perfused for 15 min with 5% (v/v) glutaraldehyde in 0.2 M sodium bicarbonate buffer (pH 9), at a flow rate of 2 ml · min^{-1}. After the tube was rinsed with 0.05 M potassium phosphate buffer (pH 8), it was perfused for 3 h at room temperature with 1 mg acetylcholinesterase in 4 ml 0.05 M potassium phosphate buffer (pH 8), with a flow rate of 5 ml min^{-1}. The unbound enzyme was removed by pumping the following solutions through the tube at 4°C at a flow rate of 5 ml min^{-1}: (1) 100 ml 0.05 M phosphate buffer (pH 8); (2) 100 ml 0.15 M NaCl; (3) 100 ml buffer consisting of 0.15 M NaCl, 2 mM phthalate, 2 mM phosphate and 2 mM borate (pH 7.0). When not in use the immobilized enzyme was stored at 4°C in the pH 7 buffer.

Rate measurements

The activity rates of the enzyme-gel slices were determined by a flow method described by Ngo et al. [13]. A slice of enzyme-containing polyacrylamide gel was suspended in 10 ml of buffered substrate solution in a temperature controlled vessel. The solution was stirred magnetically and pumped through Tygon tubing to a microvolume flow cell in a Unicam SP 1800 recording spectrophotometer. The change in absorbance at 412 nm is due to the formation of thionitrobenzoic acid from the reaction of dithiobisnitrobenzoic acid with thiocholine. The rates were calculated using $\epsilon_{412}^{1\,cm} = 1.3 \cdot 10^4\ M^{-1} \cdot cm^{-1}$.

The rates with the enzyme bound to the inner surface of nylon tube were measured by pumping the thermostatted substrate solution through the tube immersed in a temperature-controlled waterbath having the same temperature as the substrate solution. The outlet of the tube was connected to a microvolume flow cell and the absorbance was continuously recorded. All activation energies were calculated using the method of least squares analysis.

Results

Activation energies

The activation energies obtained are summarized in Table I; they were calculated from the data by the method of least squares analysis and the correlation coefficients are given in the last column.

The enzyme-slice results are for polyacrylamide discs 250 μm in thickness with an enzyme concentration of 0.012 mg per ml gel. The Arrhenius plots are shown in Fig. 1, two slopes being found at the higher substrate concentration.

The enzyme-tube results relate to tubing 50 cm in length and to two flow rates. The Arrhenius plots are shown in Figs. 2 and 3. Again, the results at the higher substrate concentration show two activation energies, at both flow rates.

pH effects with enzyme-gel sections

Acetylcholinesterase trapped in a polyacrylamide gel section of 215 μm

TABLE I

THE APPARENT ACTIVATION ENERGIES OF FREE AND IMMOBILIZED ENZYMES

Enzyme	[S]/mM	v_f/cm s^{-1}	T/°C	E/kcal · mol^{-1}	Correlation coefficient
Free	0.05	—	11—36	9.1	0.98
	2.0	—	11—36	9.4	0.98
Enzyme-gel	0.05	—	16—35	5.7	0.98
	2.0	—	16—36	9.9	0.98
	2.0	—	26—35	5.7	0.99
Enzyme-tube	0.05	8	19—40	1.7	0.98
	0.05	21	19—40	2.7	0.97
	2.0	8	16—25	5.7	0.99
	2.0	8	25—40	3.1	0.99
	2.0	21	16—30	5.5	0.99
	2.0	21	30—40	4.4	0.99

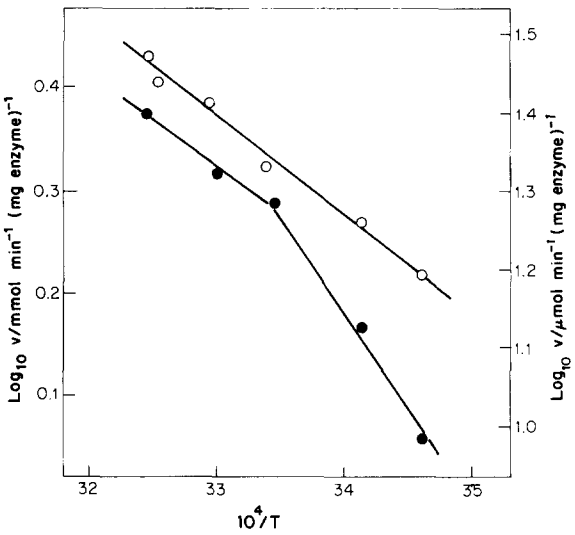

Fig. 1. Arrhenius plots of the rate of acetylthiocholine hydrolysis catalyzed by acetylcholinesterase trapped in polyacrylamide gel slices. ○, rates measured at low substrate concentration (0.05 mM): ● represents rates measured at high substrate concentration (2.0 mM). Thickness of the enzyme-gel section = 250 μm; diameter = 1 cm; enzyme concentration = 0.012 mg · cm^{-3} of gel.

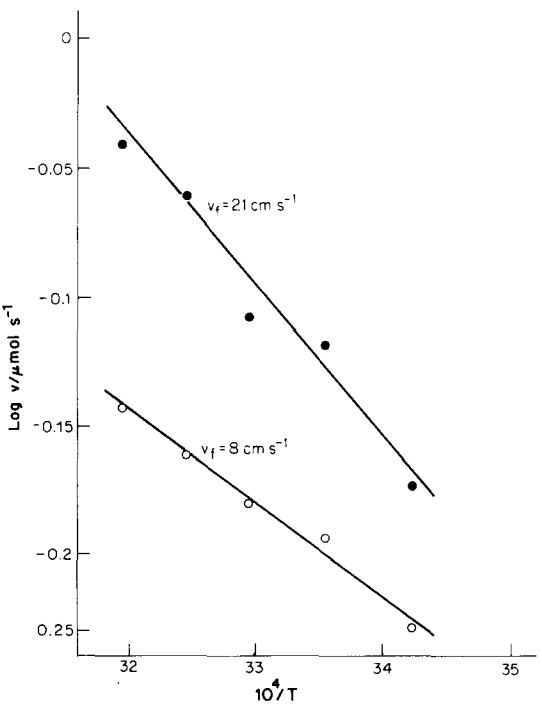

Fig. 2. Arrhenius plots of the rate of acetylthiocholine hydrolysis catalyzed by acetylcholinesterase immobilized on the inner surfaces of nylon tube. Length and inner diameter of the enzyme-tube are 50 and 0.1 cm, respectively. The flow rates, v_f, are indicated. [S] = 0.05 mM.

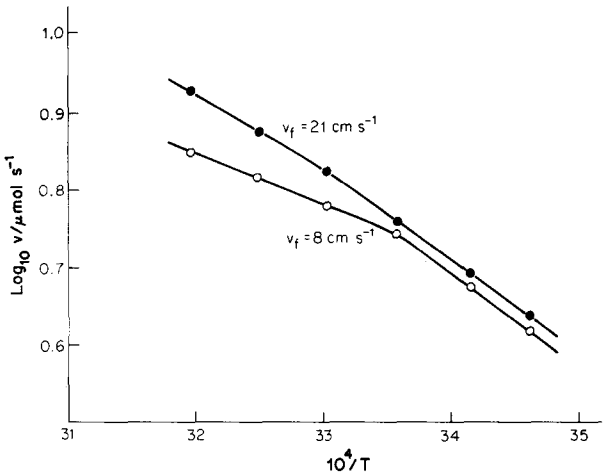

Fig. 3. Arrhenius plots of the rate of acetylthiocholine hydrolysis catalyzed by acetylcholinesterase immobilized on the inner surfaces of nylon tube. Length and inner diameter of the enzyme tube are 50 and 0.1 cm, respectively. The flow rates, v_f, are indicated. [S] = 2.0 mM.

thickness was used to study the effects of pH on the activity of immobilized acetylcholinesterase. Plots of relative activity versus pH are shown for both the free and the immobilized enzyme in Fig. 4B. The pH profile of the immobilized enzyme (closed circles in Fig. 4B) differs markedly from that of the free

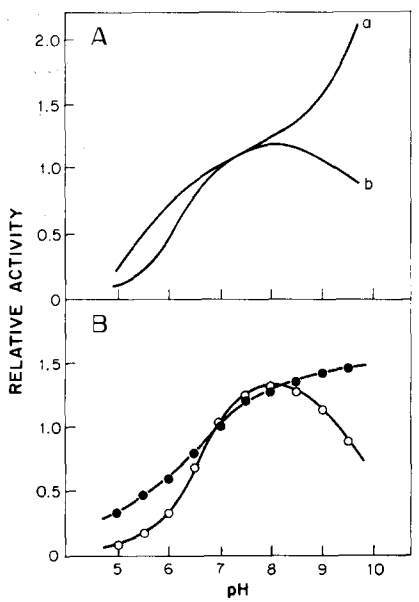

Fig. 4. Effects of pH on the activity of soluble and gel-entrapped electric eel acetylcholinesterase. A, constructed from data of Silman and Karlin [14]. Curve b is the pH vs. rate profile of acetylcholinesterase purified and solubilized from electric tissues of *Electrophorus electricus*; curve a is the pH vs. rate profile of a sub-cellular fraction rich in gel-entrapped acetylcholinesterase. All rates have been normalized to the rates at pH 7. B, pH vs. rate profile of electric eel acetylcholinesterase in soluble form (open circles) and immobilized in polyacrylamide gel (closed circles). All rates have been normalized to the rates at pH 7.

enzyme (open circles in Fig. 4B). The latter showed a characteristic bell-shaped pH curve with a pH optimum of about 8, whereas the activity of the immobilized enzyme increased continuously up to pH 9.

Fig. 4A was constructed from the data of Silman and Karlin [14]. The pH-activity profile of a solubilized and purified electric eel acetylcholinesterase is shown as curve b in Fig. 4A. The free enzyme exhibited a normal bell-shaped pH dependence. However, the pH vs. activity profile of a subcellular fraction rich in gel-entrapped acetylcholinesterase exhibited an unusual pH vs. activity curve (curve a in Fig. 4A). The activity of the gel-entrapped enzyme continuously increased as the pH of the solution increased beyond pH 9. Both free and membrane-bound enzymes were assayed in the absence of buffer, the pH of the solution being maintained by a pH-stat titrator. No pH abnormality was observed when both enzymes were assayed in a buffered solution.

Theoretical

Many treatments have been given of the kinetics of immobilized enzymes, with account taken of diffusion effects [4,5,15—23]. For an enzyme in free solution the rate is often given by

$$v = \frac{V[S]}{K_m + [S]} \tag{1}$$

where $V = k_c[E]$. The rate constant k_c relates to the breakdown of the enzyme · substrate complex and [E] is the total enzyme concentration. The Michaelis constant K_m in certain circumstances varies with temperature according to

$$K_m = a \cdot e^{\Delta E_m/RT} \tag{2}$$

where ΔE_m is an energy term. It follows that at high substrate concentrations ($[S] >> K_m$), the observed activation energy will be E_c, the activation energy relating to k_c, whereas at $[S] << K_m$ the observed activation energy is $E_c + \Delta E_m$.

An enzyme trapped in a gel has been shown [5,9,23,24] to give an equation of the form

$$v = \frac{V[S]}{K_m(app) + [S]} \tag{3}$$

where $V(= k'_c[E])$ is the limiting rate for the immobilized enzyme. The apparent Michaelis constant $K_m(app)$ is related to the intrinsic K'_m for the immobilized enzyme by

$$K_m(app) = \frac{K_m}{PF} \tag{4}$$

where P is the partition coefficient and F relates to the extent of diffusion control and is given by [8,9,23]

$$F = \frac{\tanh \gamma l}{\gamma l} \tag{5}$$

The function γ is given by

$$\gamma = \tfrac{1}{2}\left(\frac{k'_c[E]}{DK'_m}\right)^{1/2} \tag{6}$$

and l is the gel section thickness; D is the diffusion coefficient of the substrate in the membrane.

At high substrate concentrations ([S] >> K_m(app)) the rate is simply V, and diffusion effects are not involved. The rate equation then takes the form:

$$v = [E]_m A'_c e^{-E'_c/RT} \tag{7}$$

where the kinetic parameters A'_c and E'_c relate to the breakdown of the enzyme · substrate complex in the matrix. The observed activation energy is then E'_c.

At low substrate concentrations ([S] << K_m(app)) the rate is

$$v = \frac{V[S]}{K_m(app)} = \frac{V[S]PF}{K'_m} \tag{8}$$

If γl is small, F is approximately unity and there is no diffusion control. When γl is greater than about 2, F is given quite accurately by $1/\gamma l$, and therefore

$$v = \frac{V[S]}{K'_m \gamma l} = \left(\frac{4k'_c[E]D}{K'_m l^2}\right)^{1/2} P[S] \tag{9}$$

The parameters k'_c, K'_m and D can be expressed in terms of frequency factors and energies,

$$k'_c = A'_c\, e^{-E'_c/RT} \tag{10}$$

$$K'_m = A'_m\, e^{\Delta E'_m/RT} \tag{11}$$

$$D = A_D\, e^{-E_D/RT} \tag{12}$$

and therefore

$$v = \left(\frac{4[E]}{l^2}\right)^{1/2}\left(\frac{A'_c A_D}{A'_m}\right)^{1/2} \exp[-(E'_c + \Delta E'_m + E_D)/2RT] \tag{13}$$

The theory of the kinetics of the action of enzymes attached to the inner surfaces of tubes is quite complicated [20], but some general relationships have been obtained. A diffusion layer is established at the surface, and the rate with which substrate diffuses through this layer can be expressed in terms of a mass transfer coefficient k_L given by

$$k_L = \text{const.} \times \left(\frac{Dv_f}{RL}\right)^{1/3} \tag{14}$$

where D is the diffusion coefficient, v_f is the flow rate, r the radius of the tube and L its length. At high flow rates there is a reduction in the effective thickness of the diffusion layer, and little diffusion control. The apparent Michaelis constant K_m(app) is related to the value K'_m in the absence of diffusion control by

$$K_m(app) = K'_m + \frac{V}{2k_L} \tag{15}$$

so that at high flow rates K_m(app) approaches K'_m. In the absence of diffusion control the rate equation becomes

$$v = \frac{2\pi r L k'_c [E][S]}{K'_m + [S]} \tag{16}$$

At low flow rates diffusion control becomes important, and at low substrate concentrations ([S] << K_m(app)) the rate is [20]

$$v = 8.06\ (v_f D^2 r^2 L^2)^{1/3}\ [S] \tag{17}$$

It follows that, for flow systems, at higher flow rates and high substrate concentrations the activation energy will be E'_c, whereas for low flow rates and low substrate concentrations the activation energy will relate to the diffusion process.

Discussion

Enzyme-gel slice

The activation energy of 9.9 kcal · mol^{-1} found at the higher substrate concentration and in the lower temperature range is close to that for the free enzyme (9.4 kcal · mol^{-1}). This suggests (equation [7]) that the value relates to the breakdown of the Michaelis complex. The much lower value (5.7 kcal · mol^{-1}) obtained in the higher temperature range suggests that low-[S] behavior is now being observed. At the higher temperatures, the enzyme more rapidly converts the substrate to product, and the enzyme within the slice will have a low-[S] environment.

A low value (5.7 kcal · mol^{-1}) is also found with the low-[S] results and is attributed to diffusion control. If Eqn. 13 applies the observed activation energy is $\frac{1}{2}(E'_c + \Delta E'_m + E_D)$. With E'_c = 9.9 kcal · mol^{-1} it follows that $\Delta E'_m + E_D$ = 1.4 kcal · mol^{-1}. The term $\Delta E'_m$ is expected to be small, and may be positive or negative, and E_D is expected to be less than 2 kcal · mol^{-1}.

The above conclusions are consistent with the results of Buchholz and Ruth [25] with immobilized trypsin. Their apparent activation energies varied from 9.5 kcal · mol^{-1} at high substrate concentrations (similar to the value for the free enzyme) to 4.5 kcal · mol^{-1} at low substrate concentrations, the latter value indicating diffusion control.

Enzyme-tube

The low activation energies at the low substrate concentration clearly indicate diffusion control. At the higher substrate concentrations the activation energies are higher, being about one half the value for the free enzyme. This is again consistent with diffusion control, a conclusion that is confirmed by the dimensionless-parameter method of Kobayashi and Laidler [20].

pH effects

Our results with acetylcholinesterase trapped in polyacrylamide (Fig. 4B, closed circles) are consistent with results with papain in collodion [1,26] and with Silman and Karlin's results [14] with acetylcholinesterase.

Previous workers [6,14,26—28] have suggested that anomalies will arise as

a result of local pH changes in the vicinity of a gel-entrapped enzyme, as a result of substrate hydrolysis.

The intrasection pH change during hydrolysis can be estimated on the basis of the treatment of Sundaram et al. [23]. In our system acetic acid is produced and the flux J_x of the acid at a distance x from the surface of the slice is related to the local rate v_x of acid production by

$$\frac{\partial J_x}{\partial x} = -D\frac{\partial^2 c}{\partial x^2} = v_x \tag{18}$$

Writing β for $-v_x/D$ gives

$$\frac{\partial^2 c}{\partial x^2} = \beta \tag{19}$$

If c_o is the concentration at each surface, i.e., $c = c_o$ at $x = 0$ and $x = l$ the solution of Eqn. 19 is

$$c = c_0 - \tfrac{1}{2}\beta x(l - x) \tag{20}$$

At each surface

$$\frac{\partial c}{\partial x} = \pm\tfrac{1}{2}\beta l \tag{21}$$

The flux J at each surface is thus

$$J = -D\frac{\partial c}{\partial x} = \pm\tfrac{1}{2} D\beta l = +\tfrac{1}{2} v_x l \tag{22}$$

and the net flux of acid at the two surfaces is thus $v_x l$.

In our experiments $l = 0.020$ cm and the net rate of acid production per cm^2 of surface is 18.6 μmol $\cdot$ cm^{-2} $\cdot$ min^{-1}. The value of v_x is thus $18.6/60 \cdot 0.02 = 15.5$ μmol $\cdot$ cm^{-3} $\cdot$ s^{-1}, and that of β, with $D = 4 \cdot 10^{-6}$ cm^2 $\cdot$ s^{-1}, is 3.9 mol $\cdot$ cm^{-5}. If the pH is 7, $c_o = 10^{-10}$ mol $\cdot$ cm^{-3}, and the concentration at the centre of a membrane of thickness 0.02 cm is therefore, from eqn. 20,

$$c = 10^{-10} + \tfrac{1}{2} \cdot 3.9 \cdot 0.04 \cdot 0.04 = 3.1 \cdot 10^{-3} \text{ mol cm}^{-3}$$

which corresponds to a very low pH. This estimate shows that as reaction proceeds there is a very considerable reduction in pH in the interior of the support.

Acknowledgement

This work was supported by a grant from the National Research Council of Canada.

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