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Some Changes in the Reactivity of Enzymes Resulting from their Chemical Attachment to Water-Insoluble Derivatives of Cellulose

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1. Purified ficin was chemically attached to CM-cellulose, and partially purified ATP–creatine phosphotransferase was chemically attached to both CM-cellulose and *p*-aminobenzylcellulose. 2. The apparent K_m with respect to ATP and Mg^{2+} of ATP–creatine phosphotransferase was observed to increase about tenfold on attachment of the enzyme to CM-cellulose, and to increase by only 23% on its attachment to *p*-aminobenzylcellulose. 3. The reactivity of both ficin and ATP–creatine phosphotransferase with 5,5′-dithiobis-(2-nitrobenzoic acid) was observed to decrease on chemical attachment of these enzymes to water-insoluble derivatives of cellulose. With derivatives prepared from CM-cellulose, the extent of the reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) was dependent on ionic strength, but with similar derivatives prepared from *p*-aminobenzylcellulose the extent of this reaction was independent of ionic strength. 4. The effect of diffusion and electrostatic interaction of charged enzyme substrates and charged enzyme supports on the apparent K_m of a water-insoluble derivative of an enzyme is discussed. An equation is derived that satisfactorily describes the observed effects of these factors on the apparent K_m.

An enzyme that has been rendered insoluble by chemical attachment to a charged polymeric support is surrounded by a micro-environment that is defined, in part, by the nature of any charged groups on the polymeric support. For instance, there may be a significant difference between the concentration of a charged molecule in the micro-environment of the bound enzyme and its concentration in the bulk of the suspending solution. It has been suggested that this unequal distribution of ions, which has been ascribed to the electrostatic interaction between the charge field of the support and the ionic molecules in the system, can cause an alteration in the pH–activity profile (Levin, Pecht, Goldstein & Katchalski, 1964; Goldstein, Levin & Katchalski, 1964; Hornby, Lilly & Crook, 1966), apparent K_m of the bound enzyme (Goldstein *et al.* 1964; Hornby *et al.* 1966) and the difference between the reactivity of trypsin and water-insoluble trypsin with the charged pancreatic trypsin inhibitor (Levin *et al.* 1964). Although many different water-insoluble derivatives of enzymes have been prepared (Silman & Katchalski, 1966) only with a few of these has the effect on the apparent K_m been investigated.

The present work was undertaken to investigate further the effect on the apparent K_m of an enzyme with an anionic substrate when it is bound to either a polyanionic support (CM-cellulose) or an uncharged support (*p*-aminobenzoylcellulose).

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METHODS

Preparation of water-insoluble enzyme derivatives. ATP–creatine phosphotransferase (EC 2.7.3.2) was partially purified from rabbit skeletal muscle by the B procedure of Kuby, Noda & Lardy (1954). A solution of the precipitate obtained from the fractionation in 50% (v/v) ethanol was dialysed against 0·01 M-borate buffer, pH 9·0, and used in all subsequent experiments.

This enzyme preparation was chemically attached to CM-cellulose-90 (Mann Research Laboratories Inc., New York, N.Y., U.S.A.) by the method of Micheel & Ewers (1949) by using 0·5 g. of enzyme/g. of CM-cellulose-90 in the coupling reaction mixture. The method of Campbell, Leuscher & Lerman (1951) was used to couple the enzyme to

p-aminobenzylcellulose (British Drug Houses Ltd., Poole, Dorset). The reaction mixture contained 1·5g. of the enzyme and 2·3g. of the diazotized cellulose derivative Both types of water-soluble ATP–creatine phosphotransferase were washed in 1·0M-NaCl and 0·5M-$NaHCO_3$ to remove any physically attached protein (Hornby *et al.* 1966).

CM-cellulose-90–ficin and CM-cellulose-70–chymotrypsin were prepared as described by Hornby *et al.* (1966). The protein content of all cellulose–enzyme derivatives was determined by hydrolysis in 6N-HCl for 42hr. at 120°, and the liberated amino acids in the neutralized hydrolysates were determined by the method of Cocking & Yemm (1954).

Cysteine was coupled to CM-cellulose by the method of Micheel & Ewers (1949), the coupling reaction being carried out at pH 6·5 with 5·5mM-cysteine. Excess of cysteine was removed by carefully washing with N_2-flushed distilled water.

Measurement of enzymic activity. The phosphotransferase activity of ATP–creatine phosphotransferase and its water-insoluble derivatives was determined at pH 9·0 and 30° by measurement of the rate of formation of the acid-molybdate-labile phosphate (Kuby *et al.* 1954). The esteratic activity of ficin and its CM-cellulose derivative was determined as described by Hornby *et al.* (1966).

*Measurement of thiol groups with DTNB.** The method of Ellman (1959) was used, with slight modifications. Measurements on soluble enzymes were performed at 25° in phosphate buffer, pH 8·0 and *I* 0·02 or *I* 0·05, containing mM-EDTA in the presence of 0·33mM-DTNB. Where appropriate, the ionic strength of the reaction mixtures was adjusted by the addition of NaCl.

The thiol contents of water-insoluble derivatives of ficin, ATP–creatine phosphotransferase and cysteine were determined by vigorously stirring the preparations as a suspension in phosphate buffer, pH 8·0 and *I* 0·02 or *I* 0·05, in the presence of 0·33mM-DTNB. At the appropriate time, the insoluble material was removed by filtration and the E_{412} value of the filtrate measured. If desired the ionic strength was adjusted by addition of NaCl. In all cases appropriate blank determinations were performed.

* Abbreviation: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

RESULTS

Michaelis parameters of water-insoluble enzyme derivatives. ATP–creatine phosphotransferase chemically attached to CM-cellulose by the azide method of Micheel & Ewers (1949) or to *p*-aminobenzylcellulose by the diazo method of Campbell *et al.* (1951) was observed to obey Michaelis–Menten kinetics. The protein contents of the two derivatives were 2·3 and 3·0% (w/w) respectively and the maximal reaction velocities of the preparations were calculated to be 0·11 and 0·14 μmole of creatine phosphate formed/min./mg. of preparation respectively. The Michaelis constants, K_m, with respect to ATP and Mg^{2+} for these two derivatives, together with comparative data for some other enzymes coupled to insoluble supports, are set out in Table 1.

Reactivity towards DTNB. The reaction of the thiol groups of cysteine, ficin and ATP–creatine phosphotransferase, and of their corresponding water-insoluble derivatives, was shown to be directly related to the concentration of the material in the assay mixture. The numbers of moles of thiol reacting with DTNB/mg. of enzyme protein at two ionic strengths, calculated by reference to a cysteine calibration curve obtained under similar conditions, are shown in Table 2.

Table 1. *Michaelis constants of enzymes attached to solid supports and the effect of charges on the support and on the substrate*

Abbreviations: BAEE, *N*-benzoylarginine ethyl ester; ATEE, *N*-acetyltyrosine ethyl ester; BAA, *N*-benzoylarginine amide.

Enzyme	Support	Charge on support	Substrate	Charge on substrate	K'_m (M)	Reference
ATP–creatine phosphotransferase	None		ATP	−	$6·5 \times 10^{-4}$	This paper
	p-Aminobenzylcellulose	0	ATP	−	$8·0 \times 10^{-4}$	
	CM-cellulose-90	−	ATP	−	$7·0 \times 10^{-3}$	
Ficin	None		BAEE	+	$2·0 \times 10^{-2}$	Hornby *et al.* (1966)
	CM-cellulose-70	−	BAEE	+	$2·0 \times 10^{-3}$	
Chymotrypsin	None		ATEE	0	$2·7 \times 10^{-4}$	C. Money & E. M. Crook (unpublished work)
	CM-cellulose-70	−	ATEE	0	$5·6 \times 10^{-4}$	
Trypsin	None		BAA	+	$6·8 \times 10^{-3}$	Goldstein *et al.* (1964)
	Maleic acid–ethylene co-polymer	−	BAA	+	$2·0 \times 10^{-4}$	
Papain	None		BAEE	+	$1·9 \times 10^{-2}$	Silman, Albu-Weissenberg & Katchalski (1966)
	p-Aminophenylalanine–L-leucine co-polymer	0	BAEE	+	'No change from free enzyme'	

Table 2. *Reaction of the thiol groups of ATP–creatine phosphotransferase, ficin and their insoluble derivatives with DTNB*

Experimental details are given in the text.

Preparation	Thiol groups reacting in 5 min. with DTNB (mμmoles/mg. of protein)	
	At *I* 0·05	At *I* 0·65
ATP–creatine phosphotransferase	21	21
CM-cellulose-90–ATP–creatine phosphotransferase	2·0	5·0
p-Aminobenzylcellulose–ATP–creatine phosphotransferase	6·0	6·0
Ficin	46	46
CM-cellulose-90–ficin	4·9	15

Table 3. *Reaction of thiol groups of cysteine–CM-cellulose and ficin–CM-cellulose with DTNB*

The material (100 mg.) was treated as described in the Methods section, NaCl being added as required to adjust the ionic strength.

Material	Thiol groups reacting in 5 min. with DTNB (mμmoles)	
	At *I* 0·02	At *I* 0·5
CM-cellulose-70–cysteine	(1) 55	(2) 110
CM-cellulose-70–ficin	(3) 33	(4) 132
Residue from (3) washed at *I* 0·5 and washings treated with DTNB		(5) 7
Residue from (3) treated with DTNB at *I* 0·5		(6) 99

Table 4. *Effect of ionic strength on the rate of reaction between DTNB and the thiol groups of cysteine, ficin and CM-cellulose ficin*

Reactions were carried out by the method of Ellman (1959) modified as described in the Methods section. Concentrations were: DTNB, 0·33 mM; cysteine, 6·3 μg./ml.; free ficin, 339 μg./ml.; CM-cellulose–ficin, 20 mg./ml.

	$10^3 \times$ Pseudo-first-order rate constants ($sec.^{-1}$)	
	At *I* 0·02	At *I* 0·5
Free cysteine	172	221
Free ficin	88	151
CM-cellulose-70–ficin	0·4	5·5

From the results shown in Table 2 it is apparent that the attachment of these enzymes to a solid support leads to a decrease in the total of thiol groups that are accessible for reaction with DTNB in 5 min. Further, with ATP–creatine phosphotransferase and ficin attached to CM-cellulose, this effect appears to be more pronounced at low rather than high ionic strengths.

Table 3 shows that the effects of ionic strength on the reactivity of CM-cellulose–cysteine is qualitatively similar to that observed with the CM-cellulose derivatives of ficin and ATP–creatine phosphotransferase, but the overall increase in reactivity at higher ionic strengths is not so pronounced. Table 3 also contains data to indicate that with CM-cellulose–ficin the effect cannot be explained by the retention of reduced DTNB in the cellulose matrix at low ionic strengths and its liberation by 'ionic-exchange' processes at the higher ionic strengths. Finally these results also show that the thiol groups that failed to react at the low ionic strengths are still available for reaction at high ionic strengths.

Table 4 shows that the effect of ionic strength is on the velocity of the reaction between thiol and DTNB, the velocity increasing with the ionic strength. The velocity of reaction of the thiol groups in free solution is much faster than those on the solid-support derivatives. The effect of ionic strength is much more pronounced on the attached reactants than on the same reactants in free solution.

DISCUSSION

Table 1 shows that when ATP–creatine phosphotransferase is chemically attached to the polyanionic support, CM-cellulose, there is a tenfold increase in the apparent K_m of the enzyme with respect to the substrate, ATP, which is negatively charged under the conditions of the assay. This is in marked contrast with the tenfold decrease in the apparent K_m for the substrate *N*-benzoylarginine ethyl ester

(positively charged under the conditions of the assay) resulting from the attachment of ficin to the same type of support. On the other hand comparatively little change in the apparent K_m is seen when an enzyme specific for a non-charged substrate, such as chymotrypsin acting on *N*-acetyltyrosine ethyl ester, is attached to a charged support, or in the opposite situation as exemplified by the measurement made of the apparent K_m with respect to the charged substrate ATP of ATP–creatine phosphotransferase when it is attached to the uncharged support, *p*-aminobenzylcellulose.

These observations can be most readily explained by charge–charge interaction between substrate and support. Unlike charges on substrate and support would result in enhancement of the substrate concentration in the micro-environment of the bound enzyme relative to that of the substrate in the bulk of the suspending solution. Similarly, like charges on both substrate and support would result in a decrease in the substrate concentration in the micro-environment relative to that in the bulk of the solution, and if the substrate or support or both are uncharged under the conditions of the assay then there would be no difference between the substrate concentrations in the two environments. Under the various circumstances one would expect an apparent decrease, an apparent increase or little effect on the experimentally determined K_m.

A similar effect of charge–charge interaction may plausibly be invoked to explain the effects of ionic strength on thiol–DTNB interactions. At pH 8·0, where all determinations were made, the reagent, the thiol groups and the CM-cellulose support will all carry negative charges. Increasing the ionic strength will therefore be expected to screen the interacting similar charges and promote interaction. Table 4 shows that this occurs with cysteine and ficin in free solution, but that the effect is much more pronounced when ficin is attached to CM-cellulose and the concentration of negative charges in the micro-environment of the thiol group is thus increased. In free solution the reactions are virtually complete in 1–2 min. even at low ionic strengths, but the water-insoluble enzyme requires up to 2 hr. to attain equilibrium. Under the conditions used for the experiments in Table 2, therefore, only about one-third of the thiol groups react when there is unscreened charge–charge interaction. This effect disappears when there is effective charge screening (high ionic strength) or when charge–charge interactions are not enhanced by those of the support, e.g. with ATP–creatine phosphotransferase attached to the neutral support, *p*-aminobenzylcellulose. These results are important in showing that the effects of the micro-environment of a water-insoluble enzyme extend to molecules other than the substrate and raise important questions about the control of enzyme activity in biological membranes and particles. Comparable observations have been recorded for the inhibition of water-insoluble trypsin by the pancreatic trypsin inhibitor (Levin *et al.* 1964).

Although the absence of charge–charge interactions results in the absence of large changes in the apparent K_m, there remains another effect of attaching an enzyme to a solid support. According to Silman *et al* (1966), papain attached to a copolymer of *p*-aminophenylalanine and L-leucine shows no change in K_m, although they give no actual figures. However, both other enzymes in Table 1, in which charge–charge interactions between substrate and support are absent (CM-cellulose – chymotrypsin and *p* - aminobenzyl - cellulose–ATP–creatine phosphotransferase), show small increases in the apparent K_m on attachment of the enzyme to the support.

This increase is believed to be significant and is thought to be an indication of another factor affecting the substrate distribution. Although it is possibly the result of a change in conformation of the enzyme protein resulting from coupling, it seems more likely that the increase is caused by the presence of a significant diffusion-limiting layer in the neighbourhood of the attached enzyme, as was found for CM-cellulose–ficin when investigated in packed beds (Lilly, Hornby & Crook, 1966).

Goldstein *et al.* (1964) have derived an equation, which is effectively an extension of the Maxwell–Boltzmann distribution law, to describe the equilibrium distribution of a charged substrate between the charged internal micro-environment of the bound enzyme and the external solution. In deriving this equation they neglected the effects of the enzymic reaction, which would modify such a distribution by superimposing on it the effect of diffusion of the substrate into the micro-environment.

We have therefore attempted to derive an equation accounting for both electrical and diffusion effects (see the Appendix). It will be seen that this equation has a Michaelis–Menten form with the Michaelis constant, K_m, replaced by an apparent constant, K'_m, which is a function of both charge–charge interaction and diffusion. The form of the function is such that like charges on substrate and support increase K'_m and unlike charges decrease K'_m, compared with K_m. The effect of the diffusion term is always to increase the apparent K_m, the magnitude of the increase being dependent on the maximal reaction velocity of the system, the thickness of the diffusion layer and the magnitude of the diffusion coefficient of the substrate.

Although at this stage our data are not sufficiently extensive to verify quantitatively the validity of this equation, it is in good qualitative agreement with

the observations. Electrical effects are clearly dominant with the type of supported enzymes reported here, but with the preparation of more active materials or with other types of fixed enzymes diffusion may well be more important. The equation applies in general to all types of water-insoluble enzyme derivative.

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APPENDIX

An equation accounting for both electrical and diffusion effects can be derived on the basis of the Nernst–Planck equations in a manner similar to that adopted by Helfferich (1962) to describe the analogous movement of ions in ion-exchange systems.

Around each bound enzyme particle there is assumed to prevail a gradient of electrical potential, $\mathrm{grad}\,\psi$, which is generated by the charged groups on the support. In such a field, the transference of substrate from the external to the internal solutions of the bound enzyme, J_e, will be proportional to the negative gradient of the electrical potential, the concentration of substrate in the external solutions, s_o, and the electrochemical valence of the substrate, z:

$$J_e = -u_s.z.s_o.\mathrm{grad}\,\psi \qquad (1)$$

The proportionality factor in this expression, u_s, is defined as the electrical mobility of the substrate and is the rate of motion of the substrate in unit gradient of electrical potential. The electrochemical mobility is related to the diffusion constant, D, of the substrate by the Nernst–Einstein equation:

$$u_s = D.F/\boldsymbol{R}.T \qquad (2)$$

where F is the Faraday constant, $\boldsymbol{R}$ the gas constant and T is the absolute temperature of the system.

Thermal diffusion of substrate must also be considered because of the particulate nature of the bound enzyme. The rate of movement of substrate, from the external to the internal solution of the bound enzyme, due to this factor is:

$$J_d = -D.\mathrm{grad}\,(s) \qquad (3)$$

where $\mathrm{grad}\,(s)$ is the substrate concentration gradient between the internal and external solutions. To define $\mathrm{grad}\,(s)$, the diffusion layer may be approximated by a plane surface containing the bound enzyme above which is a planar layer through which the substrate must diffuse. If x is the effective thickness of this quasi-Nernst diffusion layer, and s_i is the concentration of substrate in the internal solution, then:

$$-\mathrm{grad}\,(s) = (s_o - s_i)/x \qquad (4)$$

and consequently from eqns. (3) and (4):

$$J_d = D.(s_o - s_i)/x \qquad (5)$$

Therefore the cumulative effect of J_e and J_d, describing the net rate of transference of the substrate from the external to the internal solutions, v_t, is given by:

$$v_t = J_e + J_d = \frac{D}{x}.(s_o - s_i) - \frac{D.z}{\boldsymbol{R}.T}.s_o.F.\mathrm{grad}\,\psi \qquad (6)$$

The concentration of the substrate in the internal solution, s_i, will depend on the rate at which it is being converted into product by the action of the enzyme:

$$v_r = -\frac{ds_i}{dt} = \frac{V.s_i}{s_i + K_m} \qquad (7)$$

where V is the maximum reaction velocity of the enzyme and K_m its Michaelis constant with respect to the substrate under consideration. At the steady state, where experimental observations were made, the rate of substrate transference will equal the reaction rate, i.e.:

$$v_t = v_r = v \qquad (8)$$

where v is the steady-state recorded reaction velocity. Substituting eqns. (6) and (7) into eqn. (8) it is possible to eliminate s_i to get:

$$v^2 - v.\frac{D}{x}.\left(K_m + s_o + \frac{x.V}{D} - \frac{z.x.F}{\boldsymbol{R}.T}.s_o.\mathrm{grad}\,\psi\right) + \frac{V.D}{x}.s_o.\left(1 - \frac{z.x.F}{\boldsymbol{R}.T}.\mathrm{grad}\,\psi\right) = 0 \qquad (9)$$

Eqn. (9) is of the form:

$$v^2 - v.a + b = 0$$

the roots of which approximate to $a - b/a$ and b/a (Fisher, 1964). Therefore, at equilibrium, the rate of conversion of substrate by the enzyme is given by:

$$v = a - b/a \quad \text{or} \quad v = b/a$$

where:

$$a = \frac{D}{x}.\left(K_m + s_0 + \frac{x.V}{D} - \frac{z.x.F}{R.T}.s_0.\text{grad}\,\psi\right)$$

and

$$b = \frac{V.D}{x}.s_0.\left(1 - \frac{z.x.F}{R.T}.\text{grad}\,\psi\right)$$

The first of these solutions need not be considered, since it demands that the reaction velocity of the enzyme should be infinite when the thickness of the diffusion layer, x, is zero. The second solution for the reaction velocity of the bound enzyme is:

$$v = \frac{V.s_0}{s_0 + \left(K_m + \frac{x.V}{D}\right).\left(\frac{R.T}{R.T - z.x.F.\text{grad}\,\psi}\right)} \quad (10)$$

This equation defines the apparent Michaelis constant as:

$$K'_m = \left(K_m + \frac{x.V}{D}\right)\left(\frac{R.T}{R.T - z.x.F.\text{grad}\,\psi}\right)$$

Thus if z and $\text{grad}\,\psi$ have the same sign the second expression in parentheses on the right is > 1, but it is < 1 if the signs are opposite. If either z or $\text{grad}\,\psi$ is zero the second expression in parentheses $= 1$, so that only the diffusion term remains.

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