Spectrophotometric Studies on the Interaction between Triose Phosphate Isomerase and Inhibitors

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The binding of ligands to chicken muscle triose phosphate isomerase was studied. Changes in u.v. absorbance of the enzyme were used to measure binding, and the dissociation constant was determined over a range of pH values. The ligands were 2-phosphoglycollate and *rac*-glycerol 3-phosphate (only the D-isomer, *sn*-glycerol 1-phosphate, binds appreciably). Non-linear regression was used to fit calculated curves to the experimental points and hence to compare different models. Both active sites in the dimeric enzyme probably bound 2-phosphoglycollate, without any interaction between the sites. The results of crystallographic analysis [Phillips, Rivers, Sternberg, Thornton & Wilson (1977) *Biochem. Soc. Trans.* 5, 642–647], and experiments on the ^1H , ^{13}C and ^{31}P n.m.r. of enzyme or 2-phosphoglycollate were combined with the present results to provide the basis for a model in which binding depends on glutamic acid-165 being protonated and on the ligand being fully ionized; additionally, binding affects the ionization of one histidine residue (probably histidine-100). The binding of the glycerol 3-phosphate, on the other hand, was independent of pH over the range pH 6.5–8.5 but decreased at lower pH values. This is explained on a model in which the binding of the monoanion of the ligand is markedly affected by the protonation of a residue in the enzyme, but the binding of the dianion is only slightly affected by this ionization.

Triose phosphate isomerase catalyses the inter-conversion of D-glyceraldehyde 3-phosphate and 1,3-dihydroxyacetone phosphate. The electron-density map of the chicken muscle enzyme at 0.25 nm (2.5 Å) resolution has been interpreted (Banner *et al.*, 1975), and the structure deduced forms the basis for investigations of the enzyme–substrate and enzyme–inhibitor complexes, by crystallographic methods (Phillips *et al.*, 1977).

The specificity of the enzyme limits the number of enzyme–substrate complexes available for investigation, but fortunately there are several inhibitors known, of which 2-phosphoglycollate is the most powerful (Wolfenden, 1969). Johnson & Wolfenden (1970) reported that binding of 2-phosphoglycollate to chicken muscle triose phosphate isomerase had two effects: the u.v. absorption of the enzyme was altered, and there were crystallographic changes that suggested changes in conformation of the enzyme. Glycerol 3-phosphate, a substrate analogue that is a competitive inhibitor (Burton & Waley, 1968a), had similar effects on the u.v. absorption (Johnson & Wolfenden, 1970). Both inhibitors markedly affected the p.m.r. of the enzyme, mainly by perturbing a resonance assigned to histidine-100 (Browne *et al.*, 1976).

Triose phosphate isomerase is a dimer (Johnson & Waley, 1967; Burton & Waley, 1968b), of molecular weight about 53000. When the enzyme refolds, enzymic activity is acquired as dimer is formed (Waley, 1973). Although the enzyme is a dimer, only one active site is occupied in the crystal, perhaps because an adjacent molecule is too close (Phillips *et al.*, 1977).

In the present paper we have extended the observations made by Johnson & Wolfenden (1970) to provide information about the pH-dependence of ligand binding, and about the stoichiometry and co-operativity. We find that phosphoglycollate binding demands proton uptake, a notable feature since the bound ligand is fully ionized (Campbell *et al.*, 1978). We put forward models that account for pH-dependence of binding in terms of the ionizations of specific groups.

Materials and Methods

Materials

Chicken muscle triose phosphate isomerase was prepared in the Enzyme Preparation Laboratory (Oxford Enzyme Group) under the direction of Dr.

M. P. Esnouf by the method of McVittie *et al.* (1972). Sodium *rac*-glycerol 3-phosphate and the tricyclohexylamine salt of 2-phosphoglycollate were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Dialysis tubing was thoroughly washed in the hot (McPhie, 1971).

Spectrophotometric titrations

A Zeiss PMQII single-beam spectrophotometer was used, with 1cm- or 4cm path-length cuvettes. The former contained 2.5 (or 3.0)ml with about 1.4mg of enzyme/ml, and the latter contained 10ml with about 0.35mg of enzyme/ml.

The instrument was used at maximum (15s) galvanometer damping and near-maximum amplifier sensitivity, such that the slitwidth was about 0.46mm at 289nm. Absorbance readings could be measured to an accuracy of ± 0.0005 absorbance unit. The experiments were carried out with an identical solution of enzyme in both the 'sample' and the 'reference' cell. Portions of inhibitor were added to the sample cell, and equal volumes of buffer to the reference cell. The solutions were stirred by placing the whole cell carrier on a magnetic-stirrer unit. The changes $\Delta A_{289} - \Delta A_{295}$ and $\Delta A_{288} - \Delta A_{294}$ were used to monitor binding of 2-phosphoglycollate (Fig. 1) and *rac*-glycerol 3-phosphate (Fig. 2) respectively. The enzyme concentration was measured with the use of $A_{280}^{1\%} = 12.1$ (Miller & Waley, 1971) and a molecular weight of 26600 per subunit.

Care was taken to maintain the ionic strength of the different buffers constant at 0.22M by the addition of NaCl.

Difference spectra

The difference spectrum (Fig. 5), obtained on a Cary double-beam instrument, brought about by 2-phosphoglycollate had the same form at all pH values, whereas that brought about by glycerol 3-phosphate changed progressively as the pH was lowered, and at low pH, changes at 294nm, although not as large as those at 288nm, were in the opposite direction.

Treatment of experimental titration data

Plots of extent of binding against concentration of ligand can be used to find the dissociation constant, the stoichiometry of binding and evidence of any co-operative interactions between binding sites.

The change in u.v. absorbance at a given wavelength was taken to be proportional to [SL], the amount of complex formed. Thus the fractional saturation, y , and the difference extinction coefficient, ϵ , are given by:

$$y = \frac{\Delta A - \Delta A_0}{\Delta A_\infty - \Delta A_0} \quad \text{and} \quad \epsilon = \frac{\Delta A_\infty - \Delta A_0}{S_t}$$

Fig. 1. Titration of chicken muscle triose phosphate isomerase with 2-phosphoglycollate (at 25°C and ionic strength 0.22M)

The theoretical curves were drawn with: (a) $K_d = 902 \mu\text{M}$, $10^3 \Delta A_0 = 0.1$ and $10^3 \Delta A_\infty = 44.7$; (b) $K = 1.9 \mu\text{M}$, $10^3 \Delta A_0 = 0.2$ and $10^3 \Delta A_\infty = 27.5$. These values were obtained by least-squares fitting to model (1) in the text (two independent identical binding sites). The concentration of enzyme sites was (a) $52.4 \mu\text{M}$ and (b) $10.9 \mu\text{M}$; (a) pH 8.40, 20mM-sodium borate, 1cm cuvette; (b) pH 5.14, 20mM-sodium dimethylglutarate, 4cm cuvette.

where ΔA is the difference between the absorbances at the two specified wavelengths at ligand concentration L_t and site concentration S_t , ΔA_0 is the difference at $L_t = 0$, and ΔA_∞ is the difference when $[SL] = S_t$.

The main source of experimental error was likely to be the measurement of absorbance changes.

Fig. 2. Titration of chicken muscle triose phosphate isomerase with rac-glycerol 3-phosphate (the abscissa gives the total concentration of sn-glycerol 1-phosphate) at pH 7.49 in 20 mM-triethanolamine/HCl buffer

The concentration of enzyme sites was $51 \mu\text{M}$, and a 1 cm cuvette was used. The theoretical curve was drawn with $K_d = 834 \mu\text{M}$, $10^3 \Delta A_0 = 0.2$ and $10^3 \Delta A_\infty = 41.2$.

Although the change in absorbance was small, the method was reliable, as judged by the precision of the values calculated for the dissociation constants and their agreement with values obtained by equilibrium dialysis and by kinetics (Browne & Waley, 1974; Hartman *et al.*, 1975). A FORTRAN IV program was written for non-linear regression (cf. Atkins, 1971) and run on the University ICL 1906A computer. The sum of the square of the residuals r of the m experimental points was minimized with respect to each of the n least-square parameters.

Models and parameters

Dissociation constants (K_d) were defined in terms of site concentrations (after Weber, 1975):

$$K = [L] \times \frac{[\text{free sites}]}{[\text{liganded sites}]} = \frac{[L][S]}{[SL]} \quad (1)$$

where $[L]$ is the concentration of free ligand and $[S]$ is the concentration of liganded sites. If it is assumed that each of the two identical subunits of the enzyme carries one binding site, there are several possibilities.

(a) *Independent sites.* If the two binding sites are completely independent, binding of ligand to one site has no effect on the affinity for ligand of the second site, and:

$$K_d = \frac{1-y}{y} (L_t - S_t y) \quad (2)$$

where y is the fractional saturation of binding sites, $[SL]/S_t$, S_t is the total site concentration, i.e. twice

the total enzyme concentration E_t , and L_t is the total ligand concentration.

(b) *Extreme negative co-operativity.* In this case ligand may bind with equal affinity to either site, but binding to the second site is then totally prevented:

so that:

$$\frac{K_d}{2} = \frac{1-y}{y} (L_t - 0.5 S_t \cdot y) \quad (3)$$

which is distinct from eqn. (2). For convenience, eqns. (2) and (3) may both be written in the form:

$$K_d' = \frac{1-y}{y} (L_t - \alpha \cdot S_t \cdot y) \quad (4)$$

where $\alpha = 1$ for independent sites, and $\alpha = 1/2$ for extreme negative co-operativity.

(c) *Co-operative binding: intermediate cases.* Here it is assumed that the first ligand molecule binds with equal affinity to either site, but that the second molecule of ligand then binds with a different affinity:

where $K = \sqrt{K_1 K_2}$ and $q = \sqrt{K_1/K_2}$.

If $q = 1$, $K_1 = K_2$, and eqn. (5) can be reduced to eqn. (2); as q tends to zero, $K_2 \rightarrow \infty$, and eqn. (5) becomes equivalent to eqn. (3).

Four models were fitted to each set of data, corresponding to: (1) independent sites; (2) extreme negative co-operativity; (3) unknown stoichiometry; (4) two intrinsically identical co-operative sites.

For the first three models, y is the solution (such that $0 \leq y < 1$) of the quadratic [which is eqn. (4) rearranged]: $\alpha \cdot S_t \cdot y^2 - (L_t + \alpha S_t + K_d') \cdot y + L_t = 0$. In the fourth model, y is the corresponding solution of eqn. (5). Quantities were defined individually for each model, as follows:

Model (1): $\alpha = 1$ ligand/subunit ($n = 3$ parameters)

Model (2): $\alpha = 0.5$ ligand/subunit ($n = 3$ parameters)

Model (3): $c_4 = \alpha$ ($n = 4$ parameters)

Model (4): $\alpha = 1$ ligand/subunit

$$c_3 = -\log K_d$$

$$c_4 = \log q \quad (n = 4 \text{ parameters})$$

Results and Discussion

Dissociation constants

The first model (two independent sites) fitted well to all experiments, and a satisfactory minimum was generally reached after about 15–25 iterations, but, as expected, A_∞ and K_d were quite highly correlated.

Experiments in which K_d was much less than S_i (the concentration of enzyme subunits) would have given very uncertain values of K_d . This became a problem in the experiments with 2-phosphoglycollate at low pH. For this reason, such experiments were performed in 4cm rather than 1cm cuvettes; this allowed the enzyme concentration to be decreased fourfold, to about $12\mu\text{M}$ (subunit concentration). [Concordant results were obtained at both concentrations of enzyme, and the enzyme is known to be a dimer (McVittie *et al.*, 1972) at these concentrations.] Under these conditions the relative error in K_d (as indicated by the non-linear regression program) rose to about 25% for 'small' K_d values, as opposed to about 10% for 'large' K_d values.

Stoichiometry

If the stoichiometry is to be determined at all accurately, K_d should be much smaller than S_i , and this is the exact opposite of the requirement for accurate determination of K_d . The experiments with *rac*-glycerol 3-phosphate therefore yielded no information about stoichiometry, although n.m.r. studies, where S_i is of necessity much higher (about 4mM), indicate that one molecule of glycerol 3-phosphate binds to each subunit (Browne *et al.*, 1976).

2-Phosphoglycollate, however, gave (at the lower pH values) reasonable fits to the third model. A stoichiometry of 0.82 ± 0.08 ligand/subunit (11 experiments) was thus obtained. This value is closer to 1.0 than to 0.5 (and it is easier to see reasons why it should be low rather than high), and suggests that in solution 2-phosphoglycollate binds to both the subunits, in contrast with the behaviour in the crystal referred to above.

Co-operative effects

The fourth model assumes two intrinsically identical binding sites, and allows for any interaction between them. The fit to nearly all experiments was well-behaved, and generally gave values of K_d close to those suggested by the first model, but with larger standard errors because of the uncertainty caused by introducing an extra parameter.

It may be shown that the Hill coefficient γ (being the gradient of the Hill plot of $\log[y/(1-y)]$ against $\log[L]$ at half saturation) is equal to $2q/(1+q)$. For extreme negative co-operativity $\gamma = 0$, for no co-operativity $\gamma = 1$, and for extreme positive co-operativity $\gamma = 2$ (for two sites). The experiments gave with 2-phosphoglycollate $\gamma = 0.970 \pm 0.057$ (18

experiments) and with *rac*-glycerol 3-phosphate $\gamma = 1.090 \pm 0.039$ (11 experiments) (these standard errors are calculated from the values of γ obtained from each experiment). Thus, if the stoichiometry is 1 rather than 1/2, the two sites are independent.

pH-dependence of the dissociation constants

Perhaps the most striking result of these experiments was the pH-dependence of the dissociation constants. This is apparent in the plots of $\log K_d$ against pH (Figs. 3 and 4). Above about pH 7, the slope is unity when 2-phosphoglycollate is the ligand (Fig. 3), and hence (Laskowski & Finkenstadt, 1972) one proton is taken up when the phosphoglycollate binds. In the same pH range the dissociation constant of the glycerol 3-phosphate-enzyme complex does not change with pH, so that no proton is taken up. At low pH, however, both curves change slope, indicating that binding is affected by ionizations of the enzyme or inhibitor. These results are now discussed separately for the two ligands.

2-Phosphoglycollate

The curve in Fig. 3 could be fitted with an apparent $pK_{5.5}$, well below the experimental value ($pK_{6.36}$; Campbell *et al.*, 1979) for the unperturbed pK of ligand, although it is known that it is only the fully

Fig. 3. pH-dependence of K_d for triose phosphate isomerase and 2-phosphoglycollate

The theoretical curve was calculated, as described in the text, from the double-logarithmic form of eqn. (1) of Appendix I with the values $K_6K_4/K_L = 1.1\mu\text{M}$, $pK_2 = 5.6$, $pK_7 = 6.4$ and $pK_L = 6.36$.

Fig. 4. pH-dependence of K_d for triose phosphate isomerase and rac-glycerol 3-phosphate

The theoretical curve is calculated with the least-squares parameters given in the text from the function:

$$\log K = \log K_0 + \log(1 + 10^{pK_{app.} - pH})$$

with $K_0 = 0.86 \text{ mM}$ and $pK_{app.} = 5.7$.

ionized form of 2-phosphoglycollate that is bound to the enzyme. Moreover, the pK value of a histidine residue is altered in the liganded enzyme (Browne *et al.*, 1976). The pK value of the histidine residue is raised, but the extent of the change cannot be precisely determined because it is hard to decide how much of the perturbation is to be allotted to changes in chemical shift of the resonance and how much to changes in the ionization constant. A relatively small change in pK of one residue, provisionally taken as histidine-100 (Browne *et al.*, 1976), and a large change in pK of another, taken as glutamic acid-165 (see below), result in proton uptake on binding. The pH-dependence of the dissociation constant is given by eqn. (1) of Appendix I. The non-linear regression program was used to fit the results of 18 experiments, over the pH range 5.1–8.4, to the double logarithmic form of eqn. (1); with $pK_L 6.36$, there were three independent variables. The values found, with their standard errors in parentheses, were: $K_0 K_d / K_L = 1.1 (\pm 0.2) \mu\text{M}$, $pK_2 5.6 (\pm 0.2)$ and $pK_7 6.4 (\pm 0.1)$. The values of 5.6 and 6.4 are quite possible for the pK values of the affected histidine residue in the unliganded and liganded enzyme respectively (Browne *et al.*, 1976). There is no sign in Fig. 3 of the slope lessening at pH 8.4, and so the pK of the greatly affected group seems to be perturbed by about 5 units, from ≤ 4.5 in the unliganded enzyme to ≥ 9.5 in the liganded enzyme. The binding is strong: the limiting value is $1 \mu\text{M}$ for $K_0 K_d / K_L$, the dissociation constant for the equilibrium $\text{HEHL} \rightleftharpoons \text{HE} + \text{HL}$, which applies at low pH.

The bound phosphoglycollate exists as the trianion (Campbell *et al.*, 1978), and the essential proton is taken up by a group on the enzyme. Campbell *et al.* (1978) suggested, on the basis of the crystallographic evidence (Phillips *et al.*, 1977), that the group that took up this proton was glutamic acid-165. Thus one

possibility is that the carboxylate group of 2-phosphoglycollate is hydrogen-bonded to the protonated (un-ionized) carboxy group of glutamic acid-165. This structure would account for the obligatory proton uptake: the proton taken up constitutes the hydrogen bond. The pK (< 4.5) for this enzyme group is consistent with the value (about 4) put forward by Hartman *et al.* (1975) for the active-site glutamic acid residue in the yeast enzyme.

Two experiments carried out to compare the dissociation constant from u.v. measurement with the K_i from kinetics gave a dissociation constant of $6.6 \mu\text{M}$ (range = ± 0.50) at 19°C at pH 7 in 0.1 M-triethanolamine hydrochloride, not appreciably different from the K_i obtained under these conditions (about $5 \mu\text{M}$; Hartman *et al.*, 1975). Similarly, there is little difference between the values of the dissociation constant from u.v. difference spectroscopy and from kinetics for the isomerase and phosphoglycollo-hydroxamic acid (Lewis & Lowe, 1973).

Glycerol 3-phosphate

The binding of rac-glycerol 3-phosphate was independent of pH over the range pH 6.5–8.5 (Fig. 4); the value over this range was about 0.86 mM, which is essentially the same value as is obtained by equilibrium dialysis or by kinetics (Browne & Waley, 1974); at pH 6.1 the value was 1.20 mM, and Browne *et al.* (1976) also reported a value of 1.2 mM. It is known that only the D-isomer (*sn*-glycerol 1-phosphate) will bind (Campbell *et al.*, 1979); ligand concentration is taken to be the concentration of this isomer alone. At lower pH values the dissociation constant does depend on the pH; this feature is now discussed.

The results of 15 experiments over the pH range 5.5–8.4 were fitted to the function:

$$\log K = \log K_0 + \log(1 + 10^{pK_{app.} - pH})$$

the values found were $K_0 = 0.86 (\pm 0.04) \text{ mM}$ and $pK_{app.} = 5.7 (\pm 0.06)$. The interpretation of the pH-dependence is based on the model in Appendix II, which takes into account the ionization of the phosphate group of the ligand L and the ionization of an enzyme group. The ionization of free ligand is shown as the vertical process at the back of the cube, measured by K_{12} , and the ionization of the unliganded enzyme as the horizontal process at the back of the cube, measured by K_2 ; the nature of EH is discussed shortly. Eqn. (1) in Appendix II reduces to the simple form given above, i.e.:

$$\log K = \log K_0 + \log(1 + 10^{pK_{app.} - pH})$$

if $K_{11} = K_2$ or $K_{11} = K_{12}$. Thus one possibility is that K_{11} , which is a molecular constant that represents the second stage in the ionization of the species EHLH, has the same value as the ionization of the free ligand

Fig. 5. Difference spectra for the complexes between muscle triose phosphate isomerase and (a) 1.12 mM-2-phosphoglycollate at pH 7.03, (b) 9.8 mM *sn*-glycerol 1-phosphate at pH 7 and (c) 9.8 mM-*sn*-glycerol 1-phosphate at pH 5.5

The concentrations of enzyme sites were 4.8 μ M, 4.8 μ M and 4.7 μ M in (a), (b) and (c) respectively; the buffers were 20 mM-triethanolamine/HCl at pH 7 and 20 mM-sodium 3,3'-dimethylglutarate at pH 5.5; the temperature was 25°C and the ionic strength 0.22 M.

(K_{12}); then EH, with a pK_2 of about 5.7, would represent the ionization of the histidine residue whose pK or its chemical shift (or both) was affected by ligand (Browne *et al.*, 1976). However, another, and

in some ways more attractive, hypothesis is that the ionization of glutamic acid-165 is again playing a part here, as we have suggested that it does in the binding of 2-phosphoglycollate. It turns out that the experimental points for the pH-dependence of the dissociation constant for glycerol 3-phosphate (Fig. 4) can be adequately fitted with pK_2 4.0 and pK_{11} 5.5 in eqn. (1) of Appendix II; the latter value is consistent with the evidence from the ^{31}P n.m.r. on the pH-dependence of the chemical shift of bound glycerol 3-phosphate ($pK_{11} = 5.75 \pm 0.25$; Campbell *et al.*, 1979). The value of $pK_2 = 4$ is essentially the value suggested by Hartman *et al.* (1975) for the pK of the active-site glutamic acid in the yeast enzyme. Note that the value of $pK_{11} = 5.5$ sets only an upper limit on the pK of glutamic acid-165 in the complex (pK_{11} in the model). It is only molecular constants and not group constants that can be reliably determined (Klotz, 1974; Dixon, 1976) in these situations.

The dissociation constant rises as the pH falls below 6 owing to the relative instability of the species EHLH, expressed by the relative largeness of the molecular ionization constant representing the first stage of ionization, $K_{13} + K_{15}$. This is also tantamount to saying that binding of monoanion to the protonated enzyme (EH) is markedly disfavoured, for if $K_{13} \gg K_{12}$ then $K_{16} \gg K_{17}$, as $K_{17}K_{13} = K_{12}K_{16}$. This model is also used to explain the pK obtained from the ^{31}P n.m.r. experiments on enzyme-bound glycerol 3-phosphate (Campbell *et al.*, 1979). Moreover, it seems hardly likely to be a coincidence that the pH-dependence of the inactivation of the isomerase by glycidol phosphate also gave a pK of 5.6 (Waley, 1972). The conditions required for the pH-dependence of the inactivation to give a reliable pK value (Knowles, 1976; Brocklehurst & Dixon, 1977) were probably fulfilled here.

Nature of difference spectrum

The difference spectrum brought about by the presence of 2-phosphoglycollate (Fig. 5) is a characteristic perturbation difference spectrum, i.e. one produced mainly by a small shift of the absorption bands along the wavelength axis. There are minima at 281 nm and 289 nm. These are the values expected for perturbation of tyrosine residues; Brandts & Kaplan (1973), for example, found peaks at 282 nm and 290 nm in the first derivative spectrum of ribonuclease. Perturbation of tryptophan residues produces peaks at rather longer wavelengths, e.g. 298 nm in aldolase (Crowder *et al.*, 1973); indeed, the small changes at 298 nm and 306 nm suggest that a tryptophan residue was being affected, albeit slightly. The difference spectrum that results from binding of glycerol 3-phosphate is, broadly, similar (Fig. 5). So, too, is the difference spectrum that is obtained

when the isomerase is treated with bromohydroxyacetone phosphate, an irreversible inhibitor that alkylates glutamic acid-165 (De la Mare *et al.*, 1972).

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APPENDIX I

Model for the Binding of 2-Phosphoglycolate to Triose Phosphate Isomerase

A scheme to explain the binding of 2-phosphoglycolate to triose phosphate isomerase is shown

below (Scheme 1). The enzyme (E) is shown, combined with ligand (L) or protons (H); ionizations

Scheme 1.

are 'vertical' or 'sideways', and ligand-binding is horizontal ('forwards'). We incorporate the finding, from ^{31}P n.m.r. (Campbell *et al.*, 1978, 1979), that only the fully ionized form of 2-phosphoglycollate is bound to the enzyme, i.e. L rather than HL. Thus dissociations of the liganded enzyme that give rise to HL have not been included in the scheme. K_L is the relevant ionization constant ($\text{p}K_{6.4}$) of the ligand.

Ionization of the enzyme entails protonation (with $\text{p}K$ about 6) to give HE, or (with $\text{p}K \leq 4.5$) to give EH. The ionization of this group in the liganded enzyme, however, does not occur below $\text{pH} 9.5$. It is the latter form, EH, that binds ligand, so that the liganded enzyme is present as HEHL, EHL or both, and there is little HEL or EL present. Then, if $[\text{H}^+]$ is written as h (i.e. $h = 10^{-\text{pH}}$), the dissociation constant (K) is:

$$K = \frac{([\text{E}] + [\text{HE}] + [\text{EH}] + [\text{HEH}])([\text{L}] + [\text{HL}])}{[\text{EL}] + [\text{HEL}] + [\text{EHL}] + [\text{HEHL}]}$$

The assumptions made above are that K_1 and $K_4 \gg h$ and that K_8 and $K_{10} \ll h$, so that:

$$K = \frac{K_6 K_4 \left(\frac{K_2}{h} + 1 \right) \left(\frac{K_L}{h} + 1 \right)}{K_L \left(\frac{K_7}{h} + 1 \right)} \quad (1)$$

APPENDIX II

Model for the Binding of *sn*-Glycerol 1-Phosphate to Triose Phosphate Isomerase

The effect of pH on the binding of glycerol 3-phosphate is discussed on the basis of the scheme given below (Scheme 1; opposite edges of the 'back face' show the same process). The unliganded enzyme and the free ligand are shown at the corners of the back of the cube (in their various states of ionization), and the combined species are at the corners of the front of the cube. The K values refer to ionization constants or dissociation constants. The observed equilibrium constant (K) is then given in terms of its limiting value (K_0) at high pH by:

$$K = \frac{([\text{E}] + [\text{EH}])([\text{L}] + [\text{LH}])}{[\text{EL}] + [\text{ELH}] + [\text{EHL}] + [\text{EHLH}]}$$

We now assume that $K_{13} \gg h$ (at the pH of the experiments), and put $1/K_{11} + 1/K_{14} = 1/K_{11}$, the molecular constant for the second ionization stage of the species EHLH, and so we find that:

$$K = \frac{K_0 \left(1 + \frac{h}{K_2} \right) \left(1 + \frac{h}{K_{12}} \right)}{1 + \frac{h}{K_{11}}} \quad (1)$$

At high pH, $K = K_0$, and the pH at the intersection of

Hence at high pH:

$$\log K = \log(K_6 K_4 K_2 / K_7) + \text{pH}$$

and at low pH:

$$\log K = \log(K_6 K_4) + \text{p}K_L$$

and the intersection of the lines representing the limiting slopes is:

$$\text{pH} = \text{p}K_L + \text{p}K_2 - \text{p}K_7 \quad (2)$$

If $K_7 \sim K_L$, eqn. (1) reduces to:

$$K = \frac{K_6 K_4}{K_L} \left(\frac{K_2}{h} + 1 \right)$$

and $\text{p}K_2$ is then the apparent $\text{p}K$ observed in the pH-dependence of ligand binding.

References

- Campbell, I. D., Jones, R. B., Kiener, P. A., Richards, E., Waley, S. G. & Wolfenden, R. (1978) *Biochem. Biophys. Res. Commun.* **83**, 347–352
Campbell, I. D., Jones, R. B., Kiener, P. A. & Waley, S. G. (1979) *Biochem. J.* **179**, 607–621

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Scheme 1.