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Enzyme–Substrate and Enzyme–Inhibitor Complexes of Triose Phosphate Isomerase Studied by ^{31}P Nuclear Magnetic Resonance

By IAIN D. CAMPBELL,* ROBIN B. JONES†, PETER A. KIENER† and STEPHEN G. WALEY†
**Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K., and*
†Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, U.K.

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The complex formed between the enzyme triose phosphate isomerase (EC 5.3.1.1), from rabbit and chicken muscle, and its substrate dihydroxyacetone phosphate was studied by ^{31}P n.m.r. Two other enzyme–ligand complexes examined were those formed by glycerol 3-phosphate (a substrate analogue) and by 2-phosphoglycollate (a potential transition-state analogue). Separate resonances were observed in the ^{31}P n.m.r. spectrum for free and bound 2-phosphoglycollate, and this sets an upper limit to the rate constant for dissociation of the enzyme–inhibitor complex; the linewidth of the resonance assigned to the bound inhibitor provided further kinetic information. The position of this resonance did not vary with pH but remained close to that of the fully ionized form of the free 2-phosphoglycollate. It is the fully ionized form of this ligand that binds to the enzyme. The proton uptake that accompanies binding shows protonation of a group on the enzyme. On the basis of chemical and crystallographic information [Hartman (1971) *Biochemistry* **10**, 146–154; Miller & Waley (1971) *Biochem. J.* **123**, 163–170; De la Mare, Coulson, Knowles, Priddle & Offord (1972) *Biochem. J.* **129**, 321–331; Phillips, Rivers, Sternberg, Thornton & Wilson (1977) *Biochem. Soc. Trans.* **5**, 642–647] this group is believed to be glutamate-165. On the other hand, the position of the resonance of D-glycerol 3-phosphate (*sn*-glycerol 1-phosphate) in the enzyme–ligand complex changes with pH, and both monoanion and dianion of the ligand bind, although dianion binds better. The substrate, dihydroxyacetone phosphate, behaves essentially like glycerol 3-phosphate. The experiments with dihydroxyacetone phosphate and triose phosphate isomerase have to be carried out at 1°C because at 37°C there is conversion into methyl glyoxal and orthophosphate. The mechanism of the enzymic reaction and the reasons for rate-enhancement are considered, and aspects of the pH-dependence are discussed in an Appendix.

Triose phosphate isomerase (EC 5.3.1.1) is the glycolytic enzyme that interconverts D-glyceraldehyde 3-phosphate and 1,3-dihydroxyacetone phosphate. The possibility of studying directly a productive enzyme–substrate complex has by now attracted a good deal of attention to this enzyme. The three-dimensional structure of the chicken muscle enzyme has been inferred from a 0.25nm (2.5Å)-resolution electron-density map (Banner *et al.*, 1975, 1976) and knowledge of the amino acid sequence (Furth *et al.*, 1974). Difference Fourier maps at 0.6nm (6Å) resolution have been obtained from crystals treated with dihydroxyacetone phosphate or with 2-phosphoglycollate (Phillips *et al.*, 1977). The latter, a potential transition-state analogue, is the best inhibitor of the isomerase (Wolfenden, 1970). Another inhibitor, more closely related structurally to the substrate, is D-glycerol 3-phosphate (Burton & Waley, 1968). Both these inhibitors bring about rather similar effects on the u.v.-absorption spectrum (Johnson & Wolfenden, 1970; Jones & Waley, 1979), on the $^3H/^1H$ exchange (Browne & Waley, 1974) and the ^1H n.m.r. spectrum of the enzyme (Browne *et al.*, 1976). In the present work, we have focused our attention on the ligands. We utilize the fortunate circumstance that the ligands are phosphates. By examining the ^{31}P n.m.r. spectrum of the ligands in the presence of enzyme at different pH values we obtain information on whether the monoanion of a ligand binds, and we also get some information about the kinetics of dissociation. The ionization of the ligand bound to enzyme was also studied. This ionization must be interpreted in terms of molecular (stoicheiometric) dissociation constants to obtain valid information (Jones & Waley, 1979) (see also the Appendix to this present paper). The work described below concerns three ligands, and the enzyme from two sources. The ligands are, as well as the substrate dihydroxyacetone phosphate, glycerol 3-phosphate and 2-phosphoglycollate. The enzyme was from

chicken muscle or rabbit muscle; the amino acid sequences and kinetic properties are very similar (Corran & Waley, 1973, 1975; Furth *et al.*, 1974), and there was no evidence of any significant difference between the two enzymes in the work described below.

Preliminary accounts of some of the present findings have been reported (Campbell *et al.*, 1977*a*, 1978).

Theory

Effect of chemical exchange on the n.m.r. parameters

Ligand binding. Consider the following simple binding process:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI$$

If the shifts and linewidths of inhibitor (in Hz) when bound to the enzyme and in free solution are denoted δ_{b0}, $\Delta\nu_{b0}$, δ_{f0} and $\Delta\nu_{f0}$ respectively, then the following equations, which relate these parameters to k_{-1}, are useful.

Separate resonances are observed for the bound and free species (slow exchange) if $2\pi(\delta_{b0}-\delta_{f0}) > k_{-1}$, and then:

$$\pi\Delta\nu_b = \pi\Delta\nu_{b0} + k_{-1} \quad (1)$$

$$\pi\Delta\nu_f = \pi\Delta\nu_{f0} + k_{-1}\frac{[EI]}{[I]} \quad (2)$$

where $\Delta\nu_b$ and $\Delta\nu_f$ are the observed linewidths of the bound and free resonances. Eqn. (1) is the familiar lifetime broadening equation in n.m.r. and eqn. (2) is derived from the lifetime of the I species:

$$k_{+1}[E] = k_{-1}\frac{[EI]}{[I]}$$

An average of the bound and free species is observed (fast exchange) if $2\pi(\delta_{b0}-\delta_{f0}) < k_{-1}$, and then:

$$\Delta\nu = f_b\Delta\nu_{b0} + f_f\Delta\nu_{f0} + \frac{4\pi f_f f_b(\delta_{b0}-\delta_{f0})^2}{k_{-1}+k_{+1}[E]} \quad (3)$$

where $\Delta\nu$ is the observed linewidth and f_b and f_f are the fractions of the bound and free species. The case when $[EI] = [I]$ is then simply:

$$\Delta\nu = \Delta\nu_{b0}/2 + \Delta\nu_{f0}/2 + \pi(\delta_{b0}-\delta_{f0})^2/2k_{-1} \quad (4)$$

The observed chemical shift, in the fast-exchange case, is:

$$\delta = \frac{[I]}{[I]+[EI]}\delta_{f0} + \frac{[EI]}{[I]+[EI]}\delta_{b0} \quad (5)$$

For situations other than the above limits of fast and slow exchange or of ratios of species concentration, computer analysis of more-complex equations is necessary. Useful information, however, can often be obtained from the simple equations outlined above.

Materials and Methods

Materials

Chicken muscle and rabbit muscle triosephosphate isomerases were prepared in the Enzyme Preparation Laboratory (Oxford Enzyme Group) by methods developed by Dr. M. P. Esnouf (McVitte *et al.*, 1972). Aldolase, glyoxalase I and glycerol 3-phosphate dehydrogenase were from Boehringer Corp. (London W.5, U.K.). Sodium *rac*-glycerol 3-phosphate, 2-phosphoglycollate, dihydroxyacetone and methylglyoxal were from Sigma (London) Chemical Co. (Kingston upon Thames, Surrey, U.K.). Other reagents were as described by Browne *et al.* (1976).

Preparation of samples

Samples were prepared as described in detail by Browne *et al.* (1976). The freeze-dried isomerase was chilled and dissolved in cooled $^1H_2O/^2H_2O$ (1:1, v/v) containing 0.2M-NaCl, 1mM-EDTA and 20mM-triethanolamine hydrochloride adjusted to pH7.5. The pH values quoted in this paper refer to uncorrected meter readings; the measurements were made with a Radiometer GK 2321C combination electrode, the pH-meter having been calibrated with aqueous buffers at the temperature at which the n.m.r. spectrum was being measured.

N.m.r. measurements

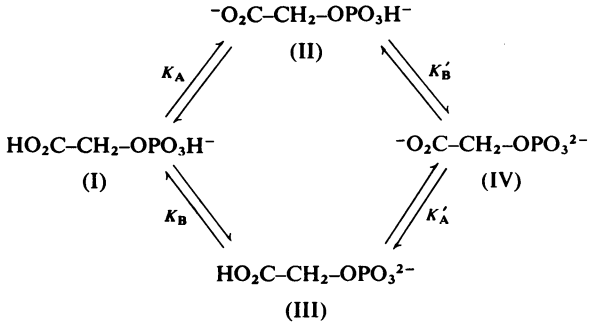
The ^{31}P n.m.r. spectra were observed at 36.4MHz on a Bruker HFX90 instrument operating in the Fourier-transform mode. ^1H decoupling irradiation was applied at all times unless otherwise stated. The accumulation time was normally about 1½h per spectrum or less, depending on the concentration of ligand. The chemical shifts are quoted upfield from trimethyl phosphate.

The ^1H n.m.r. spectra were observed at 270MHz with a long pulse to suppress the ^1H_2O resonance of the $^1H_2O/^2H_2O$ (17:3, v/v) solvent as described previously (Campbell *et al.*, 1975). The accumulation time was 35min per spectrum. The chemical shifts are quoted downfield from sodium 2,2-dimethyl-2-silapentane-5-sulphonate.

Assays

The isomerases were assayed as previously described (Waley, 1973); the $A^{1\%}_{280}$ value was taken to be 12.1 (Miller & Waley, 1971*a*). The dihydroxyacetone phosphate was determined with NADH and glycerol 3-phosphate dehydrogenase. Methylglyoxal was determined enzymically (Cliffe & Waley, 1961) or with 2,4-dinitrophenylhydrazine (Dawson *et al.*, 1969).

Paper electrophoresis at pH6.5 was used to test the inhibitors and to analyse the products of the decomposition of dihydroxyacetone phosphate; phosphates were detected by dipping the dried paper through acetone (10ml) containing 0.8ml of 12.5% (w/v) ammonium molybdate, 0.3ml of 70% (w/v) $HClO_4$ and 0.3ml of conc. HCl. The paper was then dried and exposed to sunlight or u.v. (350nm) radiation.



Preparation of dihydroxyacetone phosphate

The enzymic preparation of dihydroxyacetone phosphate from fructose 1,6-bisphosphate was the most convenient; the following procedure is a modification of the methods of Meyerhof & Lohmann (1934) and of Dr. R. N. Perham (personal communication). The use of [^{14}C]fructose bisphosphate facilitates the detection of the products in the eluates. D-Fructose 1,6-bisphosphate (1g of the tetrasodium salt and 2μCi of D-[U-^{14}C]fructose 1,6-bisphosphate) in 200ml of 10mM-ammonium formate, pH6.5, was treated with 7.5mg of rabbit muscle aldolase and 3.8mg of triose phosphate isomerase for 10min at 37°C. The pH was then lowered to 3.5 with formic acid, and the solution was extracted with 20ml of ether. The aqueous layer was applied to a column (20cm long×2cm diam.) of Dowex 1 (X4; formate form) equilibrated with 10mM-formic acid. The column was eluted with ammonium formate, pH3, 0.35M with respect to formic acid (Bartlett, 1959). The main radioactive peak contained dihydroxyacetone phosphate (883μmol); NH_4^+ ions were removed on a column (9cm long×2cm diam.) of Dowex 50 (H^+ form). The formic acid solution was 'freeze-concentrated' to give about 20ml of 36mM-dihydroxyacetone phosphate.

Results

2-Phosphoglycollate

Free 2-phosphoglycollate. The proton-decoupled ^{31}P n.m.r. spectrum of 2-phosphoglycollate consists of a single resonance whose position is pH-sensitive. The titration behaviour suggests a pK_a value of 6.36 with a chemical shift of −0.6p.p.m. in the proton-poor form and 2.62p.p.m. in the proton-rich form. The pK_a and shift difference were determined by the direct-linear-plot method of Eisenthal & Cornish-Bowden (1974) as described in Browne *et al.* (1976). The titration curve deviated at low pH, owing to the presence of another ionizable group on the molecule.

The accessible ionizations of 2-phosphoglycollate are:

The ^{31}P and ^{13}C n.m.r. results (Campbell *et al.*, 1978) are consistent with forms (II) and (IV) being the only abundant species. Potentiometric data indicated pK_a values for phosphoglycollate of 3.6 and 6.8 when measured under somewhat different conditions (Hartman *et al.*, 1975). The pK_a value of the carboxy group is perhaps surprisingly low, since the pK_a of the corresponding ionization in the sterically similar succinic acid is 5.6; the results presented here, however, are reasonably unambiguous, since the large separation between the carboxy and phosphate pK_a values allows the ionizations to be treated separately (see, e.g., Dixon, 1976).

Triose phosphate isomerase and 2-phosphoglycollate. The ^{31}P n.m.r. spectra of enzyme (about 3mM) and 2-phosphoglycollate (4mM) at several pH values are shown in Fig. 1. The sharper upfield peak is at the same position, at all pH values, as that of free phosphoglycollate; the broader downfield peak thus represents bound phosphoglycollate. This peak does not change position when the pH is varied. Therefore the bound phosphoglycollate does not change its state of ionization over the pH range 5.5–8.5. The value of the chemical shift of the bound phosphoglycollate (0.725p.p.m.) is close to the value (0.6p.p.m.) for unbound phosphoglycollate at high pH, where form (IV) predominates. Thus the bound phosphoglycollate has the phosphate group fully ionized. Moreover, the results with phospho[1-^{13}C]glycollate showed that the carboxy group of the bound phosphoglycollate was also ionized (Campbell *et al.*, 1978). Thus form (IV) represents the enzyme-bound ligand. This is an interesting result, because the weakening of the binding of 2-phosphoglycollate with increasing

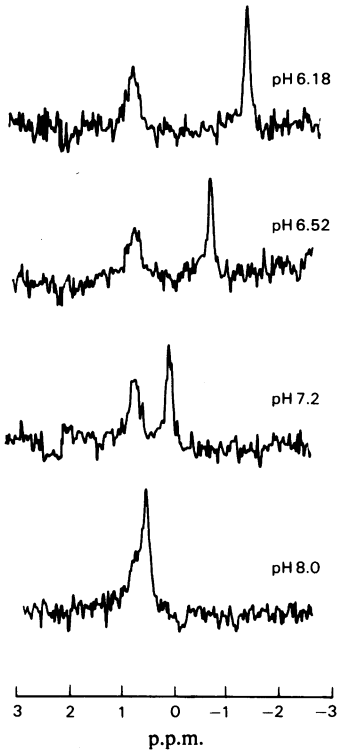


Fig. 1. *^{31}P n.m.r. spectrum of 2-phosphoglycollate (4 mM) in the presence of rabbit muscle triose phosphate isomerase (about 3 mM) in 50% ^2H_2O at 21°C*
The molarity of the enzyme denotes the concentration of subunits. In this and subsequent Figures, the pH value refers to the uncorrected pH-meter reading.

pH (Jones & Waley, 1979) shows that a proton is taken up on binding. There must therefore be a group on the enzyme that becomes protonated when phosphoglycollate binds. The nature and role of such a group are discussed below in this paper.

Information about the kinetics of the binding can also be obtained from the spectra. Since separate resonances are observed for the bound and the free ligands, the slow-exchange limit applies. At pH 8.5 the separation between the resonances was only about 5 Hz, and thus (eqn. 1) k_{-1} must be $<30 s^{-1}$. Further information is obtained from the widths of the resonances in Fig. 1. When eqn. (2) is applied, approximate values of k_{-1} can be estimated as follows. The value of [EI]/[I] calculated from the dissociation constant (Jones & Waley, 1979) is about 2 in the pH range studied, which is not inconsistent with the areas of the resonances in Fig. 1. The narrowest linewidth of any of the ligands observed under our conditions was 3 Hz, and this value is taken to be $\Delta\nu_{f0}$. The observed linewidth in the free phosphoglycollate is 4–5 Hz, and thus k_{-1} is 3–6 s^{-1}, which is compatible with the slow-exchange limit defined above. The width of the resonance assigned to the bound phosphoglycollate in Fig. 1 is about 12 Hz, so that $\Delta\nu_{b0}$ in eqn. (1) must be about 11 Hz from the above values of k_{-1} ($<6 s^{-1}$) at all pH values. The corrected values of the dissociation constant was estimated to be about 10^{-8} M (Jones & Waley, 1979) and so, if the dissociation is 4 s^{-1}, and if binding is a one-step process, the association rate constant would be $4\times10^8 M^{-1}\cdot s^{-1}$, close to the value estimated for substrate (Albery & Knowles, 1976).

rac-Glycerol 3-phosphate

Free glycerol 3-phosphate. The pair of enantiomers gives a single resonance (Fig. 2). The apparent pK_a was 6.14 in the usual solvent, 50% ^2H_2O, at 37°C, and

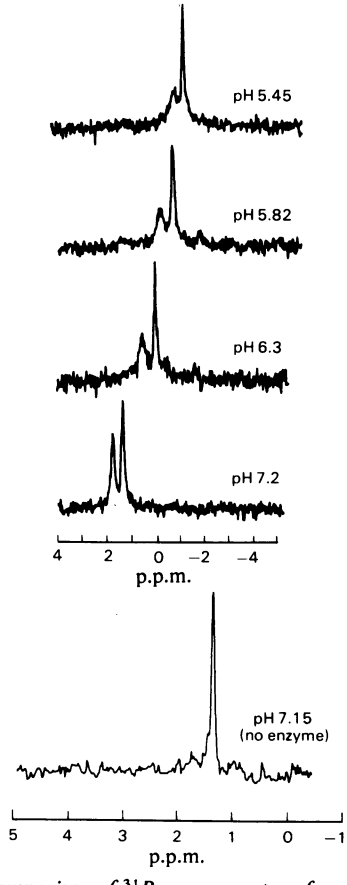


Fig. 2. *Comparison of ^{31}P n.m.r. spectra of rac-glycerol 3-phosphate (8 mM) in the presence of rabbit muscle triose phosphate isomerase (4 mM) in 50% ^2H_2O at 37°C at different pH values*
The bottom spectrum is that of 8 mM-*rac*-glycerol 3-phosphate alone, at pH 7.15.

essentially the same value was found when 25%, 75% or 100% ^2H_2O was used (the solvent also contained 0.2M-NaCl and 1mM-EDTA). The effects of the ^2H_2O on the glass electrode and on the ionization of the phosphate probably more or less cancel. The value of the true (thermodynamic) ionization constant for glycerol 3-phosphate is 6.68 (Datta & Grzybowski, 1958), and the difference of about 0.5 unit is about what is expected from the variation of the apparent ionization constant with ionic strength.

Triose phosphate isomerase and glycerol 3-phosphate. The ^{31}P n.m.r. spectrum of *rac*-glycerol 3-phosphate in the presence of the isomerase shows two resonances (Fig. 2). The positions of both resonances vary with pH (Fig. 2). The upfield member of the pair has the same chemical shift as that of the free inhibitor, at all the pH values. This resonance remained sharp but the other resonance was broadened below pH7. These effects were shown by both rabbit muscle and chicken muscle enzymes.

We first consider the origin of the two resonances. Glycerol 3-phosphate and the enzyme were in fast exchange in the ^1H n.m.r. experiments (Browne *et al.*, 1976); in that case $\delta_{b0}-\delta_{f0}\sim 43$ Hz, and thus $k_{off} > 270 s^{-1}$. The observed shift between the resonances in Fig. 2 is about 15Hz, and so slow exchange would be unlikely. Moreover, when the concentration of ligand is increased, the position of the low-field member of the pair moves upfield and merges with the other resonance. This, the upfield resonance, remains at the same position whether or not enzyme is present, and also remains sharp when enzyme is present and the temperature is lowered (Fig. 3), whereas the downfield resonance is broadened, and can no longer be seen at 1°C. These observations are accounted for by the L-enantiomer (*sn*-glycerol 3-phosphate) not binding appreciably, and indeed the position and width of the resonance of *sn*-glycerol 3-phosphate were not changed in the presence of enzyme.

Monoanion and dianion of glycerol 3-phosphate may bind to the enzyme. The pH-dependence of the resonance given by ligand in fast exchange between free and bound forms is hard to interpret directly because pH affects both binding of ligands and ionization of the phosphate. Hence the chemical shift of fully bound form was determined at several pH values. When successive amounts of *rac*-glycerol 3-phosphate were added to a fixed amount of enzyme the distance between the two resonances decreased [as stated above, the sharper resonance is assigned to the unbound L-isomer (*sn*-glycerol 3-phosphate) and the broader resonance is assigned to the D-isomer in fast exchange between free and bound forms]. The position (δ) of this broader resonance (at a given pH) is given by:

$$\delta = p_f\delta_f + p_b\delta_b$$

where p_f and p_b are the proportions of free and bound ligand and δ_f and δ_b are the chemical shifts of the free and bound ligand. Hence (since $p_f+p_b = 1$):

$$\delta-\delta_f = p_b(\delta_b-\delta_f) \quad (6)$$

Eqn. (6) was fitted to the data, p_b being calculated from the known total concentrations of enzyme and ligand and the known dissociation constant (Jones & Waley, 1979). The fit was good (Fig. 4). The values of $\delta_b-\delta_f$ at each pH and the measured values of δ_f then gave the chemical shift δ_b of the bound ligand. This varied with pH in a way that was quantitatively similar to that of the free ligand, i.e. the bound ligand titrated (Fig. 5). The change in chemical shift on ionization may be approximately assessed by the maximum slope of the curve (Browne *et al.*, 1976) as 4p.p.m., comparable with the value for free ligand.

The ionization of bound ligand is now interpreted on the basis of the model in Appendix 2 of Jones &

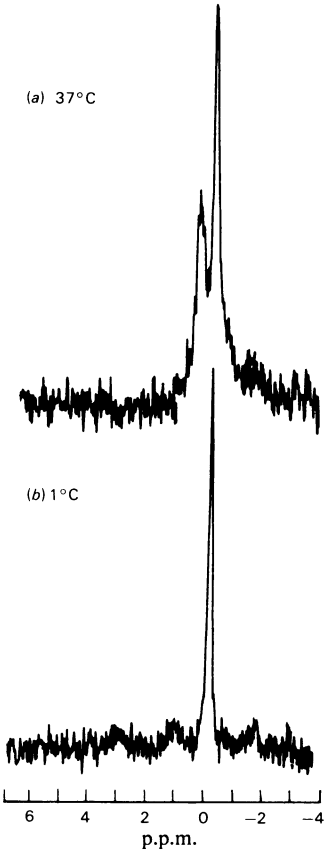


Fig. 3. *Comparison of ^{31}P n.m.r. spectrum of rac-glycerol 3-phosphate* (8mM) *and chicken muscle triose phosphate isomerase* (3mM) *at pH*6.25 *at* 37°C (*a*) *and at* 1°C (*b*)

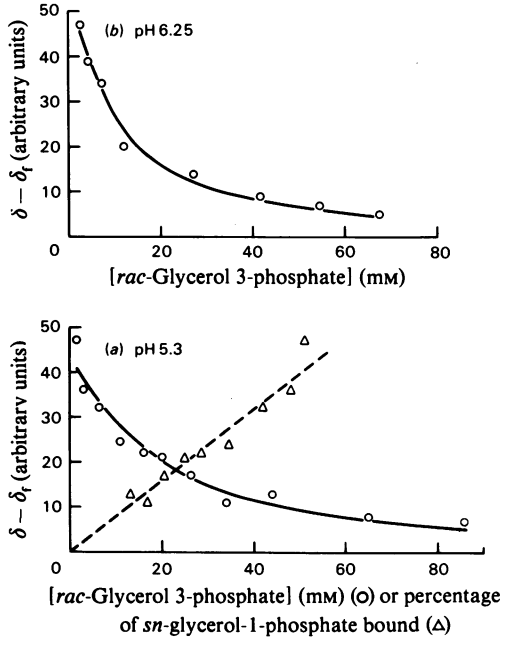
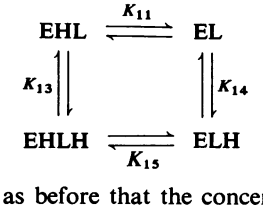


Fig. 4. *Variation of chemical shift in the ^{31}P n.m.r. spectrum during the addition of rac-glycerol 3-phosphate to chicken muscle triosephosphate isomerase* (2.7 mM) *at pH* 5.13 (*a*) *or pH* 6.25 (*b*) *in* 50% ^2H_2O *at* 37°C

The ordinate gives the value of the difference between the observed frequency and the frequency of the upfield resonance due to unbound *sn*-glycerol 3-phosphate. The line is the theoretical curve, calculated as described in the text. The proportionality is also shown in (*a*) (△) between the percentage of *sn*-glycerol 1-phosphate bound and the difference between the observed frequency and the frequency of the unbound ligand; the values on the abscissa give the percentage bound.

Waley (1979), here simplified because we need only consider bound forms:



We assume as before that the concentration of the doubly protonated complex (EHLH) is low in the pH region (≥5.3) where protonation of the (phosphate) dianion is starting, and so the chemical shift of the bound ligand at a fixed pH will be given by:

$$\delta_b = \frac{[ELH]\delta_{ELH} + [EHL]\delta_{EHL} + [EL]\delta_{EL}}{[ELH] + [EHL] + [EL]}$$

The definitions $K_{11} = h[EL]/[EHL]$ and $K_{14} = h[EL]/[ELH]$, where h is $[H^+]$, and division by [EL], then give:

$$\delta_b = \frac{\frac{h}{K_{14}}\delta_{ELH} + \frac{h}{K_{11}}\delta_{ELH} + \delta_{EL}}{\frac{h}{K_{14}} + \frac{h}{K_{11}} + 1}$$

The molecular dissociation constant (Dixon, 1976) is now introduced to replace the group constants, which are difficult to obtain: the second stage of ionization of the 'dibasic' acid EHLH is characterized by a dissociation constant K_{II}, defined as usual by:

$$K_{II}^{-1} = K_{11}^{-1} + K_{14}^{-1}$$

A mean chemical shift (δ_{II}) for the protonated bound complex is now defined by:

$$\delta_{II} = \frac{K_{14}\delta_{EHL} + K_{11}\delta_{ELH}}{K_{11} + K_{14}} = \frac{\delta_{EHL}/K_{11} + \delta_{ELH}/K_{14}}{K_{II}}$$

and hence:

$$\delta_b = \frac{\delta_{EL} + h\delta_{II}/K_{II}}{1 + h/K_{II}}$$

whence, by subtraction of δ_{EL} from both sides and rearrangement:

$$\delta_b - \delta_{EL} = \frac{h(\delta_{II} - \delta_{EL})}{h + K_{II}} \qquad (7)$$

The values of pK_{II} and $\delta_{II} - \delta_{EL}$ obtained from a program for non-linear regression (Jones & Waley, 1979) [very similar values were also obtained from a direct linear plot (Eisenthal & Cornish-Bowden, 1974; Browne *et al.*, 1976; Cornish-Bowden &

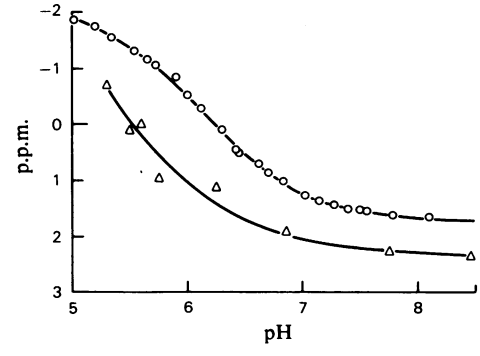


Fig. 5. *pH-titration of free* (○) *and bound* (△) *glycerol 3-phosphate*

The lines are theoretical curves, calculated with pK 6.15 and 5.75 for the free and bound glycerol 3-phosphate respectively.

Eisenthal, 1978)] were pK_{11} 5.75±0.25 and $\delta_{11}-\delta_{EL}$ 3.5±1 p.p.m. (3–5 p.p.m. from the direct linear plot); the fit is shown in Fig. 5. The change in chemical shift with pH is comparable with the change (3.8 p.p.m.) for free ligand, and ionization normally has a larger effect on the chemical shift than ligation (at high pH, the difference in chemical shift between free and bound ligand is about 0.6 p.p.m.; Fig. 5). If the effect of pH on the chemical shift is due to change of the charge on the phosphate, then the monoanion does not bind much less than the dianion. The parameters that are experimentally accessible do not directly specify the actual extent to which the monoanion binds less well than the dianion, which is expressed by K_{18}/K_0 in the 'cube' model in Appendix II of Jones & Waley (1979). Since $K_{12}K_{18} = K_0\ K_{14}$, then $K_{18}/K_0 = K_{14}/K_{12}$, and although we know the magnitude of K_{12}, we only know the limits set on K_{14} by the relationship $K_{11}^{-1} = K_{11}^{-1}+K_{14}^{-1}$ and the calculated value of K_{11}.

We now turn to the line broadening of the resonance of the glycerol 3-phosphate in fast exchange between free and bound forms. The width when 50% of the ligand was bound varied with pH. This could be accounted for by eqn. (4), on the assumption that the dissociation rate constant varied with pH, with

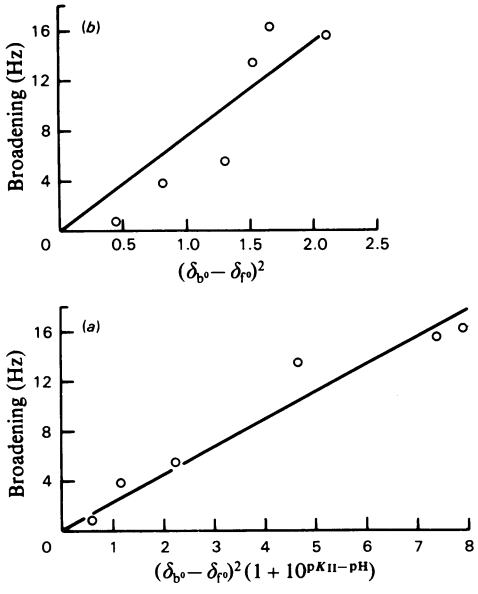


Fig. 6. *Line-broadening of the resonance of glycerol 3-phosphate when 50% of the ligand is enzyme-bound at different pH values plotted against* $(\delta_{b0}-\delta_{f0})^2(1+10^{pK_{II}-pH})$ *(a) or* $(\delta_{b0}-\delta_{f0})^2$ *(b)*

δ_{b0} and δ_{f0} are the shifts of bound and free ligand respectively, and pK_{II} is the ionization constant of bound ligand.

p*K* 5.7, dissociation taking place from the EL species. The fit to eqn. (4) was better (Fig. 6*a*) than when the dissociation rate constant was taken to be independent of pH and broadening to be due solely to the term $\delta_{b0}-\delta_{f0}$ in eqn. (4) (Fig. 6*b*). The limiting (high pH) value of the dissociation rate constant was 710 s^{-1}, much greater than the value for dissociation of 2-phosphoglycollate (3–6 s^{-1}; see above) and of the same order (10^3 s^{-1}) as the rate constant for dissociation of glyceraldehyde 3-phosphate (Albery & Knowles, 1976). If the dissociation rate constant was taken to be independent of pH (Fig. 6), its value was 306 s^{-1}. The (equilibrium) dissociation constant rises at low pH (Jones & Waley, 1979), so that the (kinetic) association constant must fall as the pH decreases whether the situation in Fig. 6(*a*) or 6(*b*) applies. The decline in the rate of association of dihydroxyacetone phosphate with enzyme is discussed by Belasco *et al.* (1978) and in the Appendix to the present paper.

The line broadening is also rather temperature-dependent, as shown in Fig. 3.

Dihydroxyacetone phosphate

Free dihydroxyacetone phosphate. All the experiments with dihydroxyacetone phosphate were carried out at 1°C, because, in the presence of relatively high concentrations of the enzyme, this compound is unstable at room temperature (Browne *et al.*, 1976) (see below). The ^{31}P n.m.r. spectrum of dihydroxyacetone phosphate shows two resonances; these arise from the keto and hydrate forms of the molecule. The resonances of the hydrogen atoms have been assigned (Gray & Barker, 1970) and the chemical shifts of the CH_2–OPO_3H^- protons are about 4.7 p.p.m. in the keto form and 3.85 p.p.m. in the hydrate form at pH 4.5 at 10°C. Selective decoupling of the ^1H resonances from the ^{31}P resonances showed that the upfield resonance was the one due to the keto form (see Fig. 7). The variation of the resonances in the ^{31}P n.m.r. spectrum with pH gave p*K* values of 5.63 and 5.78 for the keto and hydrate forms respectively.

The proportion of the keto form varies slightly with pH, and was 35–43% of the total in the present experiments (1°C, pH 5.2–7.2); other values reported are 63% at 37°C and 55% at 25°C (Gray & Barker, 1970), 55% at 20°C (Reynolds *et al.*, 1971) and 39% at 1°C (Webb *et al.*, 1977).

Dihydroxyacetone phosphate and triose phosphate isomerase. The ^{31}P n.m.r. spectrum of dihydroxyacetone phosphate (8 mM) in the presence of rabbit isomerase (about 3 mM) at pH 6.5 at 1°C consists of two resonances (Fig. 8); the upfield one is appreciably broader, and is too broad to be detectable between pH 5.8 and pH 6.3 (Fig. 9). The experimental points in the derived titration curve (Fig. 10) for the downfield resonance are fitted by the curve for the free

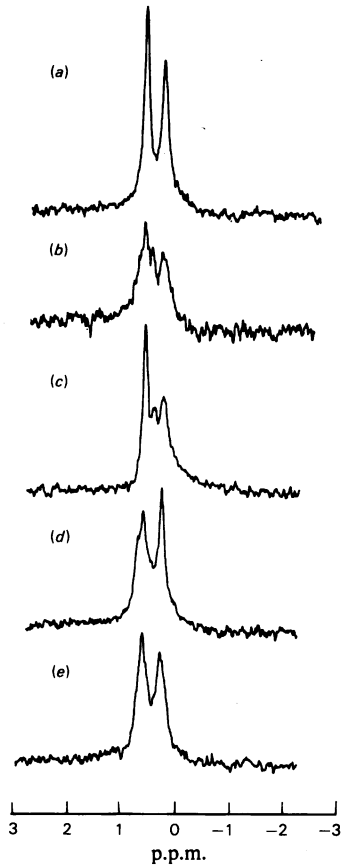


Fig. 7. ^{31}P *n.m.r. spectra of dihydroxyacetone phosphate at* 1°*C at pH* 6.4 *with selective decoupling of the hydrogen protons of the* CH_2–OPO_3^{2-} *moiety*

(*a*) Both ketone and hydrate CH_2–OPO_3^{2-} protons decoupled; (*b*) neither decoupled; (*c*) hydrate protons decoupled; (*d*) ketone protons decoupled; (*e*) frequency of 'decoupling' radiation between those used for (*c*) and (*d*).

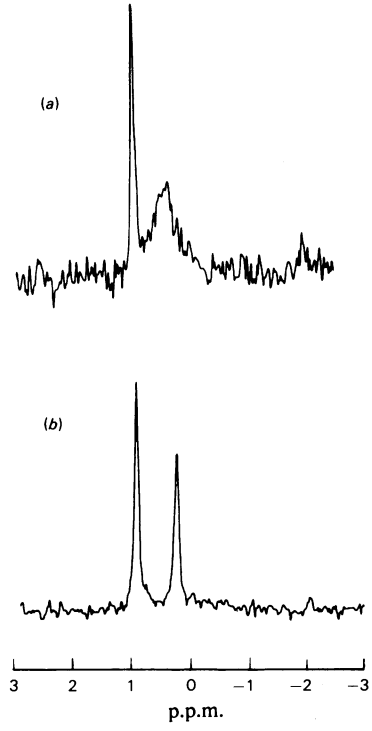


Fig. 8. ^{31}P *n.m.r. spectrum of dihydroxyacetone phosphate at* 1°*C at pH* 6.5 *alone* (*b*) *and in the presence of rabbit muscle triose phosphate isomerase* (3 mM) (*a*)

hydrate. This result is consistent with the conclusion of Reynolds *et al.* (1971) and Webb *et al.* (1977) that the hydrate does not interact with the enzyme, although weak binding (a dissociation constant of a few mM, or greater) would hardly be detected, nor would stronger binding in the perhaps unlikely event of $\delta_{bo} \sim \delta_{fo}$ and $\Delta\nu_{bo} \sim \Delta\nu_{fo}$. The binding of the keto form of dihydroxyacetone phosphate (which is the main form of bound triose phosphate) is shown by the broadening and also by a small upfield shift, of about 0.2 p.p.m., at pH 6.5. The derived pK_a was 5.85 ± 0.2 as compared with 5.63 for the unbound keto form. Examination of the effect of dihydroxyacetone phosphate on the ^1H n.m.r. spectrum of the protein showed that there was fast exchange, and this is consistent with the appearance of Fig. 9, which resembles Fig. 2 (where glycerol 3-phosphate binds in fast exchange).

The value of the dissociation constant (Albery & Knowles, 1976; Webb *et al.*, 1977) and the proportion of keto form can be combined to give a value of 52% for the fraction of the keto form of dihydroxyacetone phosphate that is bound to the enzyme. Hence $\delta_{bo} - \delta_{fo} = 0.2 \times 36.4/0.52 = 14$ Hz, and for fast exchange the dissociation rate constant $k_{off} > 88\,s^{-1}$. The evidence obtained by Albery & Knowles (1976) and Belasco *et al.* (1978) suggests that the dissociation constant of dihydroxyacetone phosphate changes little over the range pH 5.5–7, and even when the small change in the proportions of keto and hydrate forms are taken into account the fraction of the keto form bound to the enzyme is 0.5–0.55. Thus about half of the keto form is bound to the enzyme over the range pH 5.5–7, and the variation with pH of the chemical shift of the resonance due to the partly bound keto form (Fig. 10) should faithfully reflect the pH-dependence of the fully bound form. Now the change in chemical shift on protonation was about 3 p.p.m., about 10 times the change on ligation. A change of >100 Hz in the

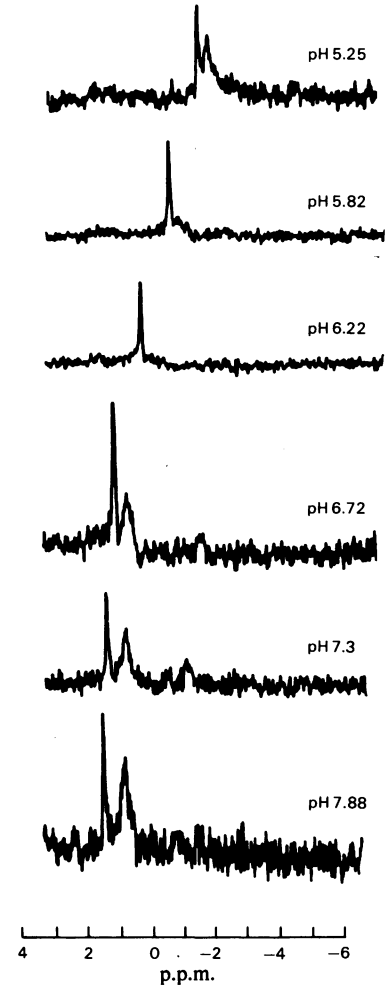


Fig. 9. *^{31}P n.m.r. spectra of dihydroxyacetone phosphate (8mM) in the presence of rabbit muscle triose phosphate isomerase (3mM) at 1°C at various pH values*

^{31}P chemical shift of a phosphoric ester, brought about by a change in pH, is either the direct effect of a change in charge on the phosphate or an indirect effect of a change in charge of one or more groups on the enzyme. A direct effect, i.e. the charge on the phosphate altering, means that the bound substrate ionizes, and that the substrate may bind as either monoanion or dianion. This possibility, mentioned above for glycerol 3-phosphate, has implications for the pH-dependence of catalysis that are discussed in the Appendix.

^{1}H n.m.r. in $^{1}H_2O$

The experiments on the ^{31}P n.m.r. of the ligands have been complemented by the use of ^{1}H n.m.r. in $^{1}H_2O$ at low field to examine changes in this region of the protein spectrum on ligation. The solvent was $^{1}H_2O$ (containing 15%, v/v, of $^{2}H_2O$) and the region 10–19p.p.m. was examined with a sweep width of 5000 or 7575Hz. Exchangeable protons that are not seen when $^{2}H_2O$ is used as solvent may be seen when $^{1}H_2O$ is used, provided that the residence time of the proton on a nitrogen atom is of the order of milliseconds (or longer). Protons of OH and SH groups usually exchange too rapidly to be seen (Glickson *et al.*, 1971) and freely exchanging protons of imidazole NH group are also in rapid exchange with the solvent and will not be seen (Patel *et al.*, 1972; Griffin *et al.*, 1973). Peptide NH groups usually give resonances at about 7.7–9.7p.p.m., the tryptophan NH group at 9.7–10.7p.p.m. and the histidine NH group at about 11–13p.p.m. (Glickson *et al.*, 1971; Griffin *et al.*, 1973; Robillard & Shulman, 1974; Campbell *et al.*, 1975, 1977*b*).

There were two resolved resonances in the spectrum of chicken muscle triose phosphate isomerase at low field, at 10.6p.p.m. and 13p.p.m. (Fig. 11), and there were also unresolved resonances at about 10.4p.p.m. The region at about 11p.p.m. is characteristic of unprotonated histidine, although some special environments are believed to be the cause of histidine resonances at about 15p.p.m. (Robillard & Shulman, 1974); protonation shifts the histidine resonance about 2p.p.m. further downfield. Thus the resonances in the isomerase may be tentatively assigned to histidine NH-group protons, in unprotonated histidine residues (10.6p.p.m.) and in a protonated (or unusually hydrogen-bonded) histidine residue at 13p.p.m. Neither the position nor (as far as could be told) the intensity of the resonances changed when the pH was varied. The resonances probably arise from non-titrating histidine residues, of which there are three in the chicken isomerase. These have been assigned to histidine-95, histidine-115 and histidine-185 (Browne *et al.*, 1976), and so there are several candidates for the resonances at about 10.5p.p.m. and at 13p.p.m.

When 2-phosphoglycollate was present there were several changes both in intensity and position (Fig. 11). Perhaps the most marked change was that the 10.6p.p.m. resonance moved downfield, and two resonances (at 12.1 and 11.7p.p.m.) appeared (Fig. 11). In the Discussion section we suggest that 2-phosphoglycollate binds so that the carboxylate group is near the $NH^{\varepsilon 2}$ group of histidine-95, and so one possibility is that the 10.6p.p.m. resonance in the unliganded enzyme should be assigned to this imidazole proton. There are, however, other possibilities, and there is not enough information to interpret the results in detail.

The changes brought about by *rac*-glycerol 3-phosphate were much harder to see, because the resonances, centred around 11.25p.p.m., were so

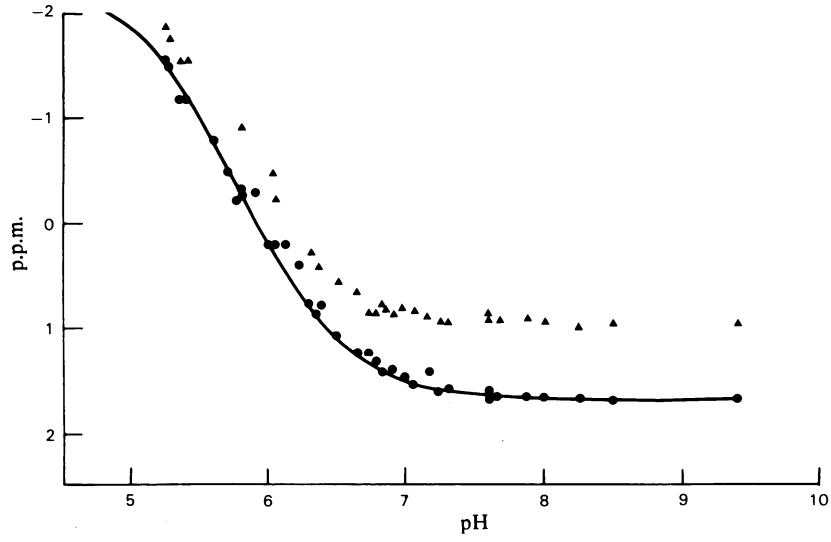


Fig. 10. *Titration data for the sharper downfield* (●) *and broader upfield* (▲) *resonances seen in the* ^{31}P *n.m.r. spectrum of dihydroxyacetone phosphate and triose phosphate isomerase* (*Fig.* 9)
The line drawn is the theoretical curve for an acid of pK_a 5.78, the pK_a of the hydrate.

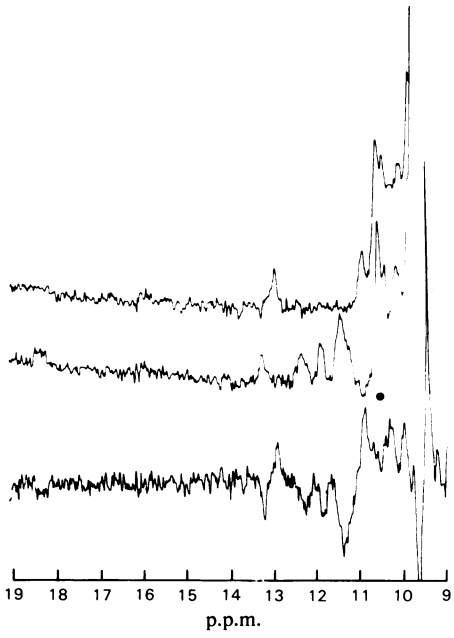


Fig. 11. ^1H *n.m.r. spectrum of chicken triose phosphate isomerase in* H_2O
The experiment was carried out in 85% H_2O at pH 7.1 at 37°C. The top of the Figure shows the spectrum of unliganded enzyme; the middle section shows the spectrum when 10mM-2-phosphoglycollate was present, and the bottom shows the difference spectrum. In the upper traces the sensitivity is expanded.

broad, partly perhaps because the lifetime of the enzyme–inhibitor complex is relatively short, of the order of milliseconds. With substrate dihydroxyacetone phosphate at 0°C, no resonances could be seen in this region; the spectrum is less well-resolved at this temperature, and the signal-to-noise ratio is unfavourable (Fig. 11). Rabbit muscle enzyme gave less reproducible results in these experiments, but the changes brought about by 2-phosphoglycollate seemed to be essentially similar to those described for the chicken enzyme.

Decomposition of dihydroxyacetone phosphate

We encountered the decomposition of dihydroxyacetone phosphate when investigating its effect on the ^1H n.m.r. spectrum of chicken muscle triose phosphate isomerase (Browne *et al.*, 1976); the decomposition products were methylglyoxal, lactic acid and inorganic phosphate. No dihydroxyacetone was detected, nor was any phosphatase activity detected in the solution of the isomerase. Hence the reaction was not hydrolysis of the dihydroxyacetone phosphate, but instead was a β-elimination of orthophosphate from the phosphoric ester of glyceraldehyde or the enediol. Although the yield of methylglyoxal was only about 20%, this yield is comparable with that obtained from the decomposition of glyceraldehyde, and is explained by the lability of methylgloxal, which decomposes to several coloured products including, perhaps, *p*-benzoquinone (Bonsignore *et al.*, 1972).

The rate of decomposition of dihydroxyacetone phosphate at 37°C was governed by the concentration of the isomerase (Fig. 12*a*). This explains why the decomposition had not been observed during work on the kinetics of the isomerization, where the concentration of the enzyme is low. The decomposition in the presence of high concentrations (25mg/ml) of enzyme was quite rapid (Fig. 12*a*). Fortunately, however, at 4°C there was little decomposition of dihydroxyacetone phosphate even in the presence of high concentrations of enzyme (Fig. 12*b*). Hence all the experiments on the ^{31}P n.m.r. of dihydroxyacetone phosphate in the presence of isomerase were carried out at 1°C, and assays carried out at the end of the experiment confirmed that there was less than 15% decomposition of the dihydroxyacetone phosphate.

The rate constant for decomposition of dihydroxyacetone phosphate in the presence of isomerase will be the weighted mean of the rate constants for the reaction of the aldehyde and the ketone (on the assumption that the hydrates of the aldehyde and ketone are unreactive); moreover, in the presence of high concentrations of enzyme, there are appreciable amounts of bound ketone, and smaller amounts of bound aldehyde; the reactivities of these are unknown. There may also be catalysis, especially by the amino groups of the enzyme; Webb *et al.* (1977) noted that the instability of dihydroxyacetone phosphate in the presence of the isomerase was no longer observed after the enzyme had been specifically inactivated. The situation is thus rather complex.

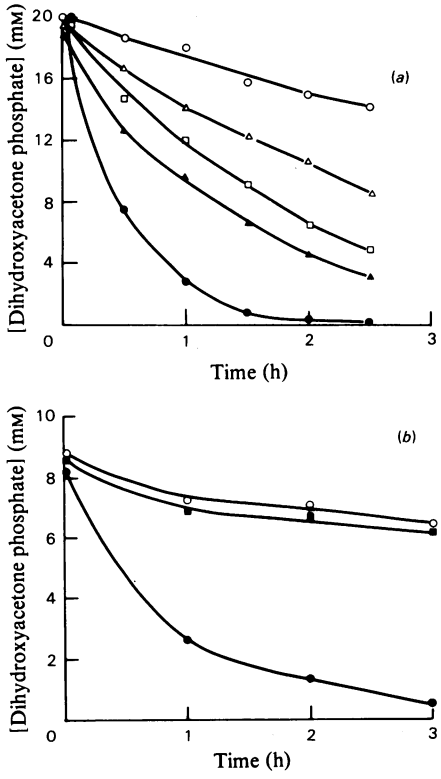


Fig. 12. *Decomposition of triose phosphates*
(*a*) Dihydroxyacetone phosphate in the presence of increasing amounts of chicken triose phosphate isomerase (mg/ml): ○, 1; △, 2; □, 4; ▲, 6; ●, 25. (*b*) Dihydroxyacetone phosphate alone (top curve), plus 25mg of isomerase/ml at 4°C (second curve), or plus 25mg of isomerase/ml at 37°C (bottom curve).

The non-enzymic conversion of dihydroxyacetone phosphate into methylglyoxal is normally slow, whereas glyceraldehyde 3-phosphate reacts more rapidly (Mel'nichenko *et al.*, 1969). Glyceraldehyde, too, decomposes, and at much the same rate as the phosphate (Fedoroňko & Königstein, 1969). In neutral solution, at 50°C, the interconversion of dihydroxyacetone and glyceraldehyde is more rapid than the formation of methylglyoxal (Fedoroňko & Königstein, 1969); similarly the interconversion of the phosphates (Hall & Knowles, 1975) is presumably more rapid than is the decomposition, since the rates of isomerization of glyceraldehyde and the phosphate are not very different. Several substances catalyse the decomposition of glyceraldehyde or its phosphate; these substances include phosphate, and, especially, lysine (Riddle & Lorenz, 1968; Mel'nichenko *et al.*, 1969; Bonsignore *et al.*, 1972), and we have confirmed this finding.

The conversion of dihydroxyacetone phosphate into methylglyoxal may be enzymic, as well as non-enzymic, but the enzyme methylglyoxal synthase is, as far as is known, restricted to bacterial cells (Hopper & Cooper, 1972; Cooper, 1974).

Discussion

Ligand binding

The results with 2-phosphoglycollate (Campbell *et al.*, 1978; Jones & Waley, 1979) show that (*a*) proton uptake accompanies binding and (*b*) the bound ligand is fully deprotonated. Hence the proton is taken up by a group on the enzyme. Campbell *et al.* (1978) suggested that this group was glutamate-165, and so the proton taken up may form a hydrogen bond between the carboxy group of glutamic acid-165 and the carboxylate group of phosphoglycollate. The proton taken up may be regarded as the counterpart of the *pro-R*-hydrogen atom on C-1 of dihydroxyacetone phosphate that is labilized in the enzymic reaction, and thus 2-phosphoglycollate is indeed somewhat akin to the postulated transition states of the catalysed reaction. The downfield resonance in the ^{1}H n.m.r. spectrum in water (Fig. 11) suggests the possibility of a histidine residue forming a

hydrogen bond with the 2-phosphoglycollate, and the crystallographic results (Browne *et al.*, 1976; Phillips *et al.*, 1977) suggest the proximity of histidine-95.

The marked pH-dependence of the binding of inhibitors with carboxy groups, in contrast with the binding of inhibitors (or substrate) lacking such a group, has also been observed with glucose 6-phosphate isomerase (Bruch *et al.*, 1973; Chirgwin & Noltmann, 1975; Chirgwin *et al.*, 1975). Perhaps an enzymic carboxy group plays a part here too (Shaw & Muirhead, 1976, 1977).

For the other inhibitor of triose phosphate isomerase that we have studied, glycerol phosphate, there is no information from crystallography. Structurally D-glycerol 3-phosphate (*sn*-glycerol 1-phosphate) resembles both substrates, since the C-1 CH_2OH moiety is present in one substrate and the C-2 CH_2OH moiety in the other. D-Glycerol 3-phosphate binds to the isomerase about as tightly as dihydroxyacetone phosphate does. If the large change in chemical shift of the bound ligand with pH (Fig. 5) is attributed to a change in charge of the phosphate, bound ligand ionizes and both monoanion and dianion may bind. The same considerations probably apply to the experiments with the substrate (Fig. 10). It may be relevant that Birdsall *et al.* (1977) found that both the monoanion and the dianion of 2′-AMP bound to dihydrofolate reductase. On the other hand, Belasco *et al.* (1978) concluded, from experiments with the isomerase and dihydroxyacetone sulphate, that monoanions bound at least 100-fold more weakly than dianions, which entails a $pK<4$ for the bound substrate. If this is so, then the effect of pH on the chemical shift of bound ligand cannot be the straightforward one of a change in charge of the phosphate, but must be an indirect effect mediated by groups on the enzyme. Further work is needed to decide between these possibilities. The model given in the Appendix shows how the results from kinetics, as explained by Belasco *et al.* (1978), can be treated slightly differently to take into account the binding of the monoanion of the substrate.

Catalysis

C–H bond fissions, key steps in the isomerization of triose phosphates, characteristically proceed much more slowly than O–H or N–H bond fissions do. Reasons for the difference include the need for both solvent reorganization and structural reorganization in C–H bond fissions (Bell, 1973, 1975). The 'two-anion' mechanism for such reactions postulates formation of a (weakly) hydrogen-bonded complex as the first stage; fission of the C–H bond, the rate-determining step, leads to formation of an unstable species with charge largely localized on carbon atoms, i.e. a carbanion (Bordwell & Boyle, 1975). This species is described as 'singly solvated', because there is now a strong hydrogen bond between the conjugate acid of the attacking base and the carbanion. Since solvation is of overwhelming importance in ionic reactions, a key feature in the action of the isomerase may consist of especially favourable interactions in the 'singly solvated' carbanion. The hydrogen bond between the carboxylate group of 2-phosphoglycollate and the carboxy group of glutamic acid-165 that we postulate on the basis of our experiments is the counterpart of the strong hydrogen bond in the 'singly solvated' carbanion. Moreover, if histidine-95 also interacts (as is suggested by the ^1H n.m.r. spectrum in water), then there might well be a 'doubly solvated' carbanion in the enzymic reaction. Such additional stabilization of an unstable intermediate may indeed be one of the factors that make this enzyme such an efficient catalyst.

The support of the Medical Research Council is gratefully acknowledged. We thank Professor J. R. Knowles for details of unpublished work and for helpful comments. This is a contribution from the Oxford Enzyme Group, and it incorporates ideas derived from the crystallographic analysis performed by Professor D. C. Phillips and his co-workers.

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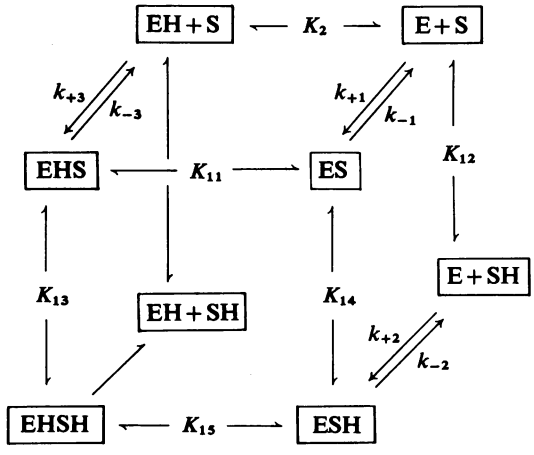
APPENDIX

pH-Dependence of an Enzymic Reaction when the Ionization of Both the Substrate and the Enzyme Have to be Taken into Account

The purpose of this Appendix is to show how the pH-dependence of an enzymic reaction can be interpreted on the 'cube model' with proper allowance being made for the ionization of both substrate and enzyme. The application to catalysis by triose phosphate isomerase is essentially a modification of the treatment by Belasco *et al.* (1978).

The interplay of ionization and dissociation is illustrated by the cube with the species at the corners and the processes along the edges (Scheme 1). Ionization of the substrate is shown vertically, ionization of the enzyme horizontally and dissociation in the third direction. The enzyme (E) may combine with substrate (S) or with protonated substrate (SH) to give complexes ES or ESH respectively, and protonated enzyme (EH) may also combine with substrate to give complex EHS or protonated substrate to give complex EHSH. No simpler model takes account of these four modes of combination, all of which have to be reckoned with when protonation of enzyme and substrate affects dissociation or association. Now note that ionization of the doubly protonated enzyme–substrate complex EHSH (in the bottom left-hand front corner of the cube) can be described in terms of group dissociation constants or molecular (stoicheiometric) dissociation constants K_I and K_{II} (Edsall & Wyman, 1958; Klotz, 1974). Usually, only the latter can be reliably determined (Dixon, 1976). The two kinds of constants are here related as follows:



Scheme 1.

$$K_I = K_{13} + K_{15}, \qquad K_{II}^{-1} = K_{11}^{-1} + K_{14}^{-1}$$

We now assume that, where the effects of decreasing pH are only starting to come into play, there is so little of the doubly protonated complex EHSH that its presence may be neglected. We also assume that proton transfers are fast enough for the concentrations of the differently charged species to be in their equilibrium ratios. The rate constant for dissociation (k_{off}) is then given by:

$$k_{off} = \frac{k_{-1}[ES] + k_{-2}[ESH] + k_{-3}[FHS]}{[ES] + [ESH] + [EHS]}$$

Now $K_{14} = h[ES]/[ESH]$ and $K_{11} = h[ES]/EHS]$ where $h = [H^+]$, and so division throughout by [ES] gives:

$$k_{off} = \left(k_{-1} + \frac{k_{-2}h}{K_{14}} + \frac{k_{-3}h}{K_{11}}\right) \Big/ \left(1 + \frac{h}{K_{II}}\right)$$

i.e.:

$$k_{off} = k_{-1} + \left(1 + \frac{h}{k_{-1}K_{14}/k_{-2}} + \frac{h}{k_{-1}K_{11}/k_{-3}}\right) \Big/ \left(1 + \frac{h}{K_{II}}\right)$$

Now from thermodynamics $k_{-1}K_{14}/k_{+1} = k_{-2}K_{12}/k_{+2}$, i.e.:

$$k_{-1}K_{14}/k_{-2} = K_{12}k_{+1}/k_{+2},$$

and

$$k_{-1}K_{11}/k_{+1} = k_{-3}K_2/k_{+3}$$

i.e.

$$k_{-1}K_{11}/k_{-3} = K_2k_{+1}/k_{+3}$$

and hence:

$$k_{off} = k_{-1}\left(1 + \frac{h}{K_{12}k_{+1}/k_{+2}} + \frac{h}{K_2k_{+1}/k_{+3}}\right) \Big/ \left(1 + \frac{h}{K_{II}}\right)$$

The treatment by Belasco *et al.* (1978) is now followed in assuming that (1) $k_{+1} \geqslant k_{+3}$, (2) $k_{+1} \gg k_{+2}$ and (3) $K_2 \gg h$, and hence:

$$k_{off} = k_{-1}/(1 + h/K_{II}) \qquad (1)$$

Eqn. (1) is of exactly the same form as that obtained by Belasco *et al.* (1978), but the interpretation here is less restricted. The ionization constant governing the pH-dependence is a molecular ionization constant: it represents a joint ionization. Bound substrate may ionize, as it seems to do (see the Results section), and the monoanion of dihydroxyacetone phosphate (protonated substrate SH in the model) may bind. Moreover, it is an oversimplification to talk of the pK of glutamic acid-165 (EH in the model) being raised to (say) 6 in the complex, because the constant (K_{II}) obtained from the experimental results relates to the joint ionization (compare the discussion of the ionization of bound glycerol 3-phosphate in the Results section).

The pH-dependence of the association rate con-

stant (k_{on}) on the present assumption that $K_2 \geqslant h$ (which means that [EH]/[E] is low) is governed by ionization of the substrate, and hence:

$$k_{on} = k_{+1}/(1+h/K_{12})$$

The pH-dependence of $k_{cat.}/K_m$ gives K_{12}, and depends on the substrate rather than the enzyme (Belasco *et al.*, 1978), in contrast with the pH-dependence of $k_{cat.}$, which gives pK_{11}. Thus the pH-dependence of K_m is governed by K_{11}/K_{12}. For chicken or rabbit muscle triose phosphate isomerase $K_{11}/K_{12} \sim 1$, and so K_m depends little on the pH (Plaut & Knowles, 1972; Hartman *et al.*, 1975). However, K_m varies markedly with the ionic strength (Hartman *et al.*, 1975), an observation that has escaped comment. This is presumably due to an effect on K_{12}. Thus increase of ionic strength decreases K_m by favouring ionization of the free phosphate; there may be little effect on K_{11} if ionization of the complex is more affected by interaction with protein groups than the medium. For yeast triose phosphate isomerase, the pH-dependence of $k_{cat.}$ gives pK 4.6 (Hartman & Ratrie, 1977), lower than the values for the chicken or rabbit muscle enzymes; there is not enough known about the yeast enzyme to be able to suggest a reason, but it is known to be unusual in being stable at pH 4.

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