pH-Dependence of the Triose Phosphate Isomerase Reaction

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(Received 16 March 1972)

The pH-dependences of the kinetic parameters k_{cat} and K_m for the triose phosphate isomerase reaction were determined in each direction. Apparent $\text{p}K_a$ values of 6.0 and 9.0 are observed in the dependences of k_{cat}/K_m . The pH-dependences of k_{cat} are sigmoid, with apparent $\text{p}K_a$ values of about 6.0. The results are interpreted in terms of a single base on the enzyme providing an efficient proton-shuttling mechanism for the isomerization.

One of the many pieces of information relating to the interaction of an enzyme with its substrate is the variation of the kinetic parameters with pH. Although pH-dependence results have often been developed to unwarranted conclusions and the meaning of 'apparent' $\text{p}K_a$ values has been stretched mercilessly, it remains true that the dependence of the rate of enzyme-catalysed reactions on pH is an important parameter that must be accommodated by any complete proposal of mechanism. Some interesting and controversial problems of mechanistic interpretation in recent years have hung on the variation of kinetic and spectroscopic properties with pH, as exemplified by the question of assignment of the apparent $\text{p}K_a$ values of α -chymotrypsin (Birktoft *et al.*, 1970), and of papain (Lowe, 1970). Discussion continues on these matters partly because we have little quantitative information as yet on the macroscopic $\text{p}K_a$ values of linked (e.g. hydrogen-bonded) systems such as the His-Cys pair in papain or the Ser-Asp-His-Ser quartet in chymotrypsin, but also because the variations in intrinsic $\text{p}K_a$ of a group on a protein surface are still so ill-defined. For any enzyme the mechanistic details of which are under study, and for which a high-resolution crystal structure exists, there is fundamental information to be gained from a knowledge of the pH-dependence of the individual kinetic parameters. These dependences must be consistent with the mechanistic proposals, and must be interpretable in the knowledge of the three-dimensional structure of the enzyme.

For the enzyme under scrutiny here, triose phosphate isomerase, there is an additional reason why knowledge of the pH-dependence is important. When the substrate dihydroxyacetone phosphate, stereospecifically tritiated on C-3, is converted into the product D-glyceraldehyde 3-phosphate, essentially all the ^3H is lost to the solvent (Rieder & Rose, 1959). There is only about 2% transfer of ^3H from C-3 of dihydroxyacetone phosphate to C-2 of glyceraldehyde phosphate (S. G. Maister & J. Herlihy, un-

published work). This is illustrated in Scheme 1. Since k_{cat} for this reaction is about 470s^{-1} /subunit (see Table 1), the 2% of ^3H transfer requires that the rate of dissociation of the enzyme conjugate acid (B-T in Scheme 1) must be at least $50 \times 470\text{s}^{-1}$; i.e., $23\,500\text{s}^{-1}$. Now, Eigen (1964) has shown that all common bases, with oxygen, nitrogen or sulphur centres, combine with protons in aqueous solution with a second-order rate constant around $10^{10}\text{M}^{-1}\cdot\text{s}^{-1}$. Only carbanions behave differently. This requires that an enzymic acid group that dissociates with a rate of $20\,000\text{s}^{-1}$ must have a $\text{p}K_a < 6$, unless proton tunnelling is invoked. This problem is even more acute in the 'reverse' reaction of triose phosphate isomerase, since the k_{cat} for glyceraldehyde phosphate is about 4700s^{-1} at 30°C . The extent of ^3H transfer in this direction is not yet known, but this information, coupled with the pH-dependence results presented here, will clearly be of great importance in delineating the rates of proton-transfer steps in enzyme-catalysed reactions.

The present paper reports the pH-dependence of the Michaelis parameters for the triose phosphate isomerase-catalysed reaction in each direction, together with results of the control experiments needed to validate the assay methods over the pH range used.

Experimental

Materials

Triose phosphate isomerase. This was isolated from chicken breast muscle as described in the preceding paper (Putman *et al.*, 1972). The concentration of enzyme solutions was determined by measuring E_{280} , assuming $E_{280}^{0.1\%}$ of 1.21 for a 10mm light-path. A subunit molecular weight of 25000 was assumed and kinetic parameters quoted relate to a single subunit.

D-Glyceraldehyde 3-phosphate dehydrogenase and α -glycerophosphate dehydrogenase. These were obtained from Sigma (London) Chemical Co. Ltd.,

Scheme 1. Reaction of specifically tritiated dihydroxyacetone phosphate with triose phosphate isomerase

London S.W.6, U.K. The units of activity used are as defined by the supplier [Sigma (London) Chemical Co., catalogue]. Trace contamination by triose phosphate isomerase was removed by treatment of the dehydrogenase (2ml of a 1mg/ml solution) with chlorohydroxyacetone phosphate (10 μ l of a 5mM solution in ether) (de la Mare *et al.*, 1972). After incubation for 5min at room temperature, the enzymes were dialysed exhaustively against 13mM-triethanolamine-HCl buffer, pH7.5, at 4°C. This removes both the excess of inactivator and the (NH₄)₂SO₄ in which the enzymes are stored.

NAD⁺ and NADH. These were obtained from Sigma Chemical Co. An extinction of 6.22 $\times$ 10³ M⁻¹ cm⁻¹ for NADH was assumed (Horecker & Kornberg, 1948).

Dihydroxyacetone phosphate dimethyl ketal. This was prepared as the bis-monocyclohexylamine salt as described by Ballou & Fischer (1956). It had m.p. 183°C [Ballou & Fischer (1956) give 183–185°C]. T.l.c. on cellulose plates in methanol-pyridine-water-NH₃ (4:2:2:1, by vol.) showed a single phosphate-containing component (Hanes & Isherwood, 1949), of *R_F* 0.49. Concentrations of dihydroxyacetone phosphate were determined by enzymic reduction with α -glycerophosphate dehydrogenase and NADH.

DL-Glyceraldehyde 3-phosphate. This was obtained from Sigma Chemical Co. as the diethyl acetal monobarium salt. Concentrations of the D-enantiomer were determined enzymically by using either glyceraldehyde phosphate dehydrogenase, NAD⁺ and arsenate, or isomerase, α -glycerophosphate dehydrogenase and NADH.

Buffers. These were prepared with A.R.-grade reagents where available, and deionized water. The ionic strength was adjusted to 0.1 with NaCl, except

for the carbonate buffers with dihydroxyacetone as substrate for which the ionic strength was between 0.1 and 0.2. The buffer systems used were as follows: $\beta\beta$ -dimethylglutaric acid-NaOH (0.04M, pH5.3–6.7); triethanolamine-HCl (0.1M, pH6.6–8.6); Na₂CO₃-NaHCO₃ (0.04M, pH8.6–9.8).

Methods

U.v. absorption measurements. At fixed wavelengths these were taken on a Unicam SP.1800 instrument. All kinetic runs were carried out on a Unicam SP.800 spectrophotometer equipped with an SP.850 scale expander coupled to a Sunvic 10S chart recorder. The temperature of the cell block was maintained at 30 $\pm$ 0.5°C with a constant-temperature circulating water bath. Solutions were contained in glass cells of 10mm light-path.

pH measurements. These were made at 30°C with a Radiometer TTT1c pH-meter fitted with a pHA 630 scale-expander attachment, standardized at 20°C against standard buffer solutions from British Drug Houses Ltd., Poole, Dorset, U.K. *pK_a* determinations were performed at 30°C on the above instrument, coupled to a Titrator SBR 2c. Substrate concentrations were 0.5–2.5mM. The *pK_a* values of dihydroxyacetone phosphate and of glyceraldehyde 3-phosphate were 6.22 and 6.49 under these conditions, though in the presence of 100mM-NaCl (i.e. the ionic strength of the kinetic experiments) these values fell to 6.00 and 6.30 respectively.

Circular-dichroic spectral measurements. These were made at room temperature with a Dichographe II Roussel-Jouan instrument. The wavelength range was 260–225nm. Isomerase (100 μ l, 0.36mM, in 20mM-triethanolamine-HCl buffer, pH7.4) was added to 3.0ml of 20mM-glycine-NaOH buffers at

pH values of 8.03, 9.21 and 9.94. Duplicate spectra were obtained and no differences in the spectra at different pH values could be detected.

Kinetic studies. The conditions for kinetic work were as follows.

With dihydroxyacetone phosphate as substrate, the cuvette contained: buffer solution (1.0 l); EDTA (5 mM); sodium arsenate (6 mM); NAD^+ (1 mM); D-glyceraldehyde 3-phosphate dehydrogenase (0.17 mg/ml); dihydroxyacetone phosphate (0.2–2.0 mM); and triose phosphate isomerase (40 ng/ml) to initiate the reaction. The total volume was 3 ml. Dihydroxyacetone phosphate is normally stored at pH 4.5, and the pH was raised to that of the experiment before use. Solutions of dihydroxyacetone phosphate were found to be stable at pH 9.0 and 0°C for at least 21 h. At the pH extremes (less than pH 6.5 or greater than 9.8) twice the concentration of coupling enzyme mentioned above was used. For experiments at pH 9.82 and 9.86, the isomerase concentration was raised to 81 ng/ml.

With glyceraldehyde phosphate as substrate, the cuvette contained: buffer solution (1.0 l); EDTA (5 mM); NADH (0.2 mM); α -glycerophosphate dehydrogenase (0.017 mg/ml); D-glyceraldehyde 3-phosphate (0.2–1.0 mM); and triose phosphate isomerase (1 ng/ml) to initiate the reaction. The total volume was 3 ml. The pH of stock solutions of DL-glyceraldehyde 3-phosphate was raised to that of the run before use. For experiments above pH 8.63, the concentration of α -glycerophosphate dehydrogenase was 0.034 mg/ml, and above pH 9.0 it was 0.068 mg/ml.

Initial rates were measured at each substrate concentration from the appearance or disappearance of NADH, measured at 340 nm. The pH values of the reaction mixtures were determined after completion of each experiment.

Calculation of kinetic parameters. The kinetic parameters k_{cat} (calculated per subunit) and K_m were obtained from an unweighted least-squares analysis of plots of v_0 versus $v_0/[S_0]$ (the gradient of which gives K_m) and $[S_0]$ versus $[S_0]/v_0$ (the gradient of which gives k_{cat}), where v_0 is the initial velocity and $[S_0]$ is the initial substrate concentration (see Dowd & Riggs, 1965). All values relate to a single subunit. Errors quoted in Tables 1 and 2 and shown in Figs. 4, 5 and 6 are precision estimates only, and indicate the s.d. of the least-squares line. $\text{p}K_a$ values were calculated by the approach described previously (Cornish-Bowden & Knowles, 1969).

Results

pH-stability of triose phosphate isomerase

The stability of the enzyme as a function of pH is shown in Fig. 1. Triose phosphate isomerase

Fig. 1. Relative enzymic activity remaining after incubation of triose phosphate isomerase in buffer of appropriate pH at 38°C for 6 h

For details see the text.

(7.6 $\mu\text{g/ml}$) was incubated for 6 h at 38°C in buffers of appropriate pH, after which the remaining enzymic activity was assayed at pH 7.5. The extremes of pH used in kinetic measurements were 5.37 and 9.86, and at these pH values, negligible catalytic activity is lost (this is true even at enzyme concentrations of 40 ng/ml) during the time of a kinetic run (between 5 and 10 min at 30°C).

Validity of the coupled-enzyme assays

To test that the observed rates of production or oxidation of NADH truly represent the rate of the triose phosphate isomerase-catalysed reaction, the variation in initial rate with concentration of coupling enzyme was determined. When conditions were found under which the overall reaction was limited by the isomerase reaction, this was further checked by studying the initial rate as a function of isomerase concentration at the highest concentrations of substrate to be used in the kinetic runs.

Results for the variation of the rate of dihydroxyacetone phosphate isomerization as a function of glyceraldehyde phosphate dehydrogenase concentration are shown in Fig. 2(a), and as a function of isomerase concentration at high substrate concentrations, in Fig. 2(b). Similar results in the reverse direction (with glyceraldehyde phosphate as substrate) are shown in Figs. 3(a) and 3(b). The arrows in Figs. 2 and 3 indicate the concentrations of reagents used in subsequent experiments. From plots of this kind, 'safe' concentrations of all species in solution were determined, and are listed in the Experimental section. The fact that relatively massive excesses of coupling dehydrogenase did not affect the reaction

Fig. 2. Test of the coupled-enzyme assay: dihydroxyacetone phosphate as substrate

(a) Initial rate of NADH production as a function of glyceraldehyde 3-phosphate dehydrogenase concentration. Assays contained: dihydroxyacetone phosphate (0.28 mM); triose phosphate isomerase (40 ng/ml); 0.1 M-triethanolamine-HCl buffer, pH 7.51, at 30°C; and glyceraldehyde 3-phosphate dehydrogenase (approx. 40 units/mg, at 25°C). (b) Initial rate as a function of triose phosphate isomerase concentration. Assays contained: dihydroxyacetone phosphate (1.31 mM); glyceraldehyde 3-phosphate dehydrogenase (0.17 mg/ml); and 0.1 M-triethanolamine-HCl buffer, pH 7.51, at 30°C. Reagent concentrations used are shown by the arrows.

rate after the plateau had been reached rules out any complications from enzyme-enzyme interactions.

Similar checks on the validity of the assay procedure were performed at the extremes of the pH range, around pH 5.5 and 10. It was found necessary at the high pH extreme to increase the coupling-enzyme concentration for reactions in either direction, and also at the low pH extreme when dihydroxyacetone phosphate was the substrate. The instability of NAD^+ and of NADH at high and low pH values respectively (Lowry *et al.*, 1961) necessitated small corrections at the pH extremes. A small 'blank' rate

Fig. 3. Test of the coupled-enzyme assay: glyceraldehyde 3-phosphate as substrate

(a) Initial rate of NADH oxidation as a function of α -glycerophosphate dehydrogenase concentration. Assays contained: D-glyceraldehyde 3-phosphate (0.717 mM); triose phosphate isomerase (3.4 ng/ml); 0.1 M-triethanolamine-HCl buffer, pH 7.69, at 30°C; and α -glycerophosphate dehydrogenase (approx. 100 units/mg, at 25°C). (b) Initial rate as a function of triose phosphate isomerase concentration. Assays contained: D-glyceraldehyde 3-phosphate (0.717 mM); α -glycerophosphate dehydrogenase (0.017 mg/ml); and 0.1 M-triethanolamine-HCl buffer, pH 7.69, at 30°C. Reagent concentrations used are shown by the arrows.

of production of material absorbing at 340 nm, arising from NAD^+ decomposition at high pH, was subtracted from the initial-velocity measurements with dihydroxyacetone phosphate as substrate in this pH region. A similar small amount of NADH decomposition was observed at low pH values for reactions with glyceraldehyde phosphate as substrate. In no case did the instability of a coenzyme lead to its concentration affecting the reaction rate.

pH-dependence of k_{cat} and K_m

At each pH value, initial rates were obtained over a tenfold range of substrate concentration when dihydroxyacetone phosphate was substrate, and a fivefold range when glyceraldehyde phosphate was substrate. Under the conditions used, cleanly linear

Table 1. *Kinetic parameters for the triose phosphate isomerase-catalysed reaction of dihydroxyacetone phosphate*

For experimental details see the text.

Buffer	pH	$10^{-7} \times k_{\text{cat.}}/K_m^*$ ($\text{M}^{-1} \cdot \text{min}^{-1}$)	K_m^* (mM)	$10^{-4} \times k_{\text{cat.}}$ (min^{-1})
β, β -Dimethylglutarate	5.60	0.58 ± 0.01	1.05 ± 0.05	0.61 ± 0.01
	5.91	0.75 ± 0.01	1.54 ± 0.11	1.16 ± 0.05
	6.23	1.17 ± 0.02	1.66 ± 0.10	1.98 ± 0.06
	6.62	1.42 ± 0.06	1.59 ± 0.22	2.32 ± 0.18
Triethanolamine	6.60	1.66 ± 0.01	1.51 ± 0.03	2.50 ± 0.03
	6.80	1.72 ± 0.04	1.39 ± 0.13	2.55 ± 0.10
	7.51	1.87 ± 0.01	1.57 ± 0.03	2.92 ± 0.04
	8.05	1.80 ± 0.04	1.52 ± 0.09	2.72 ± 0.08
	8.57	1.69 ± 0.05	1.70 ± 0.12	2.67 ± 0.16
Carbonate	8.68	1.29 ± 0.02	2.07 ± 0.11	2.67 ± 0.09
	9.31	0.69 ± 0.02	3.42 ± 0.54	2.61 ± 0.41
	9.82	0.29 ± 0.01	6.25 ± 2.89	1.95 ± 0.76
	9.86	0.30 ± 0.01	7.64 ± 1.99	2.36 ± 0.56

* Uncorrected for arsenate inhibition (see text).

Table 2. *Kinetic parameters for the triose phosphate isomerase-catalysed reaction of D-glyceraldehyde 3-phosphate*

For experimental details see the text.

Buffer	pH	$10^{-8} \times k_{\text{cat.}}/K_m$ ($\text{M}^{-1} \cdot \text{min}^{-1}$)	K_m (mM)	$10^{-5} \times k_{\text{cat.}}$ (min^{-1})
β, β -Dimethylglutarate	5.37	1.12 ± 0.03	0.42 ± 0.03	0.47 ± 0.01
	5.77	2.51 ± 0.13	0.72 ± 0.09	1.84 ± 0.14
	6.08	4.06 ± 0.04	0.48 ± 0.01	1.96 ± 0.04
	6.53	5.24 ± 0.08	0.42 ± 0.03	2.21 ± 0.08
Triethanolamine	6.62	5.47 ± 0.18	0.43 ± 0.04	2.40 ± 0.12
	7.40	6.61 ± 0.16	0.39 ± 0.02	2.60 ± 0.06
	8.07	6.36 ± 0.05	0.44 ± 0.01	2.80 ± 0.02
	8.63	5.80 ± 0.02	0.49 ± 0.01	2.81 ± 0.01
Carbonate	9.11	4.54 ± 0.11	0.67 ± 0.04	3.07 ± 0.10
	9.34	2.92 ± 0.09	0.80 ± 0.09	2.40 ± 0.18
Glycine	9.47	2.98 ± 0.06	0.86 ± 0.06	2.60 ± 1.03
Carbonate	9.49	1.53 ± 0.06	1.42 ± 0.70	2.48 ± 1.02
	9.63	1.49 ± 0.06	1.84 ± 0.42	2.95 ± 0.49

double-reciprocal plots are obtained (see Putman *et al.*, 1972). The values of the parameters $k_{\text{cat.}}$ (calculated per subunit) and K_m for reaction in the forward and reverse directions are listed in Tables 1 and 2. From plots of $k_{\text{cat.}}/K_m$ versus pH (Fig. 4), apparent $\text{p}K_a$ values of 6.05 and 9.05 (with dihydroxyacetone phosphate as substrate) and of 6.0 and 9.2 (with glyceraldehyde phosphate as substrate) are observed. Corresponding plots of $k_{\text{cat.}}$ versus pH (Fig. 5) yield apparent $\text{p}K_a$ values of 6.0 and 5.9 respectively.

Discussion

Validity of the assay

The assay for triose phosphate isomerase used in this work involves the coupling of the isomerization with the oxidation of NADH or the reduction of NAD^+ (Warburg & Christian, 1943; Beisenherz, 1955). The validity of coupled-enzyme assays depends on the product of the target reaction being removed by the coupling enzyme as fast as it is formed. Bergmeyer (1963) has considered the theoretical

Fig. 4. Plots of $k_{cat.}/K_m$ versus pH for the triosephosphate isomerase-catalysed reaction of dihydroxyacetone phosphate (a) and of D-glyceraldehyde 3-phosphate (b)

For experimental details see the text. Δ , β , β -Dimethylglutarate buffer; $\blacksquare$, triethanolamine buffer; $\bullet$, carbonate buffer. The curves are theoretical, for pK_a values of 6.05 and 9.05 (a) and 6.0 and 9.2 (b).

basis of such assays and concludes that the observed rate is within 1 % of the rate of the target reaction only if the effective activity of the coupling enzyme is a thousand times greater than that of the target enzyme. This condition is often very difficult to meet, and could require massive concentrations of coupling enzyme in some circumstances. The situation is not, however, as depressing as the above implies. As has been discussed by McClure (1969), it is not uncommon in coupled-enzyme assays to observe at the start of the reaction a period during which the rate increases to a steady value, and then a relatively long period during which the rate is constant, ending in the fall-off in rate as substrate or cofactor is consumed. In the region of linearity, the steady-state concentration of the product of the target reaction is constant, and the observed rate is the same as the rate of the target

Fig. 5. Plots of $k_{cat.}$ versus pH for the triose phosphate isomerase-catalysed reaction of dihydroxyacetone phosphate (a) and of D-glyceraldehyde 3-phosphate (b)

For experimental details see the text. Δ , β , β -Dimethylglutarate buffer; $\blacksquare$, triethanolamine buffer; $\bullet$, carbonate buffer. The curves are theoretical, for pK_a values of 6.0 (a) and 5.9 (b).

reaction. Only if the steady-state concentration of the intermediate species is significant relative to the initial substrate concentration are errors introduced. In the present experiments it was rarely possible to observe a significant acceleration phase, and the concentration of intermediate species was always very low. From experiments of the type illustrated in Figs. 2(a) and 3(a), 'saturating' concentrations of coupling enzyme and of cofactors can be determined. The most economical check on the validity of the assay is the dependence of initial rate on target-enzyme concentration [see Figs. 2(b) and 3(b)], which must be performed at the highest substrate concentrations to be used (or more strictly, under substrate conditions

leading to the maximum rate of initial-product formation).

Another possible source of error in the assay for triose phosphate isomerase relates to the fact that the free triose phosphates are in equilibrium in solution with forms that are not substrates for the enzyme. Thus Trentham *et al.* (1969) have shown that only 3% of glyceraldehyde phosphate is in the form of the free aldehyde in neutral aqueous solution at 20°C, and that it is the free, unhydrated, monomeric aldehyde that is the substrate for the enzyme. Similarly, Reynolds *et al.* (1971) find that only 55% of dihydroxyacetone phosphate is in the free keto form that can be utilized by triose phosphate isomerase. Since the isomerase-catalysed reaction is so fast, it is important to establish that the measured rates of reaction do not relate to the rates of production of the unhydrated substrate forms of the triose phosphates.

Consider the simplified scheme:

where S is the free carbonyl form of the substrate, $\text{S} \cdot \text{H}_2\text{O}$ is the inactive hydrate, [E] is the free enzyme concentration, P is the product, and k_{+h} and k_{-h} relate to the hydration and dehydration reactions. It can be shown that:

$$[\text{E}_{\text{total}}] = [\text{E} \cdot \text{S}] + \frac{K_m \cdot k_{+h}}{\frac{[\text{S} \cdot \text{H}_2\text{O}] \cdot k_{-h}}{[\text{E} \cdot \text{S}]} - k_{+2}} \quad (1)$$

Now if $k_{+2} \ll [\text{S} \cdot \text{H}_2\text{O}] \cdot k_{-h} / [\text{E} \cdot \text{S}]$, and if we consider the situation with glyceraldehyde phosphate as substrate (which, of the two reaction directions, is the more likely for substrate dehydration to be rate-limiting) so that $[\text{S}_0] \approx [\text{S} \cdot \text{H}_2\text{O}]$, we have:

$$[\text{E}_{\text{total}}] = [\text{E} \cdot \text{S}] \left(1 + \frac{K_m \cdot k_{+h}}{[\text{S}_0] k_{-h}} \right) \quad (2)$$

Since v , the observed rate, is $k_{+2} \cdot [\text{E} \cdot \text{S}]$, eqn. (2) becomes the Michaelis equation, with $k_{+2} = k_{\text{cat}}$, and $K_m \cdot k_{+h} / k_{-h}$ = the observed K_m . That is, the Michaelis equation will be obeyed, with the correct intrinsic k_{cat} , and a K_m that is higher than the intrinsic one (relating to the free aldehyde form of the substrate) by the factor k_{+h} / k_{-h} . The condition that $k_{+2} \ll [\text{S} \cdot \text{H}_2\text{O}] \cdot k_{-h} / [\text{E} \cdot \text{S}]$ is equivalent to the condition that $k_{\text{cat}} / k_{-h} \ll [\text{S}_0] / [\text{E}_{\text{total}}]$, since $k_{+2} = k_{\text{cat}}$, [E·S] cannot be larger than $[\text{E}_{\text{total}}]$, and $[\text{S}_0] \approx [\text{S} \cdot \text{H}_2\text{O}]$. Now, k_{cat} for glyceraldehyde phosphate is 4700 s^{-1} (Fig. 5) and k_{-h} is 0.087 s^{-1} at 20°C (Trentham *et al.*, 1969), so k_{cat} / k_{-h} is about 50000.

Therefore, for Michaelis kinetics to be followed, $[\text{S}_0] / [\text{E}_{\text{total}}]$ must be greater than 50000. (This is an upper limit, since k_{-h} at 30°C will be higher than the number used.) In the present experiments, this limit has not been approached, and the lowest value of $[\text{S}_0] / [\text{E}_{\text{total}}]$ used for kinetic runs involving glyceraldehyde phosphate was 5×10^6 .

A further question on the validity of the assay is the use of racemic glyceraldehyde phosphate as substrate. It is commonly assumed that the L-enantiomer has no effect on the assay, and is not a significant inhibitor. Certainly L- α -glycerophosphate does not inhibit the enzyme, whereas the D-enantiomer does (K_i 0.115 mM; Burton & Waley, 1968). The agreement between the equilibrium constant determined directly starting from dihydroxyacetone phosphate, and that determined from the individual kinetic parameters by using the Haldane relationship (Burton & Waley, 1968; Putman *et al.*, 1972), does not necessarily confirm the assumption that the unnatural L-isomer of glyceraldehyde phosphate is without effect on the kinetic parameters for triose phosphate isomerase, even though this isomer is not present in the determination of equilibrium constant. If the L-isomer is a competitive inhibitor of the enzyme and is always equimolar with initial substrate concentration, the value of k_{cat} / K_m (used for the Haldane relationship), is unchanged. Direct evidence that it is justified to neglect the presence of L-glyceraldehyde phosphate comes from the near-identity of K_m values for DL-glyceraldehyde phosphate and its optically pure D-enantiomer (S. J. Putman, unpublished work).

It was noticed by Burton & Waley (1968) that under the normal conditions of assay with dihydroxyacetone phosphate as substrate, the arsenate ion present competitively inhibits the isomerase and leads to an erroneously high K_m value, and a consequent error in the overall equilibrium constant determined from the Haldane relationship. The possibility that the inhibition by arsenate is pH-dependent is unlikely, since the two relevant $\text{p}K_a$ values for arsenate are 6.77 and 11.53 (Bjerrum *et al.*, 1957). The upper value is outside the pH range of the kinetic measurements reported, and the lower value occurs in a pH region where K_m is constant for the reactions in each direction (see Fig. 6). It should be noted that sulphate ion is also an inhibitor of triose phosphate isomerase (Turner *et al.*, 1965) and it is important to remove this ion from the coupling dehydrogenases that are normally supplied as $(\text{NH}_4)_2\text{SO}_4$ suspensions.

pH-dependence

The pH-dependences of k_{cat} / K_m for the triose phosphate isomerase-catalysed reaction in each direction are shown in Fig. 4. These follow the theoretical titration curves for two groups reasonably closely. Significant specific effects of buffer ions are not

Fig. 6. Plots of K_m versus pH for the triose phosphate isomerase-catalysed reaction of dihydroxyacetone phosphate (a) and of D-glyceraldehyde 3-phosphate (b)

For experimental details see the text. Δ , β,β -Dimethylglutarate buffer; $\blacksquare$, triethanolamine buffer; $\bullet$, carbonate buffer. The curves are theoretical, for pK_a values of 9.2 (a) and 9.25 (b).

apparent, though with dihydroxyacetone phosphate as substrate it appears that the enzyme is more active in triethanolamine buffers than in β,β -dimethylglutarate or in carbonate. A pronounced effect was observed in tris buffers, the enzyme being about half as active in this system; these results are not included in Tables 1 and 2. The possibility of specific buffer effects in a pH-dependence study is normally unavoidable, and puts a limit on the precision of the apparent pK_a values derived from such results. From Fig. 4, pK_a values of 6.05 and 9.05 (with dihydroxyacetone phosphate as substrate) and 6.0 and 9.2 (with glyceraldehyde phosphate) are observed. It has been pointed out by Peller & Alberty (1959) that for mechanisms where only one ionization state of enzyme and of enzyme-substrate complexes are directly interconvertible, the pH-dependence of k_{cat}/K_m gives pK_a values of the free enzyme and free substrate, and the pH-dependence of k_{cat} gives pK_a

values of the enzyme-substrate complex whose breakdown is rate-limiting. Most steady-state pH-dependence work has relied heavily on this simple interpretation, even though it is very rare that one can be assured that it is applicable. As has been stressed by Schmidt & Westheimer (1971) 'the pH-rate profile cannot be used directly to determine pK_a 's on the enzyme when different steps in the overall process depend on different levels of protonation'. The exclusive operation of the simplified pathway assumed by Peller & Alberty (1959) is extraordinarily difficult to show experimentally, and until this has been done, assignments of pK_a values must remain tentative. With this caveat in mind, we can examine the consequences of such an assumption, in the present case.

Apparent pK_a values in k_{cat}/K_m may relate to ionizations in free enzyme or in free substrate. Under the conditions of the present experiments, the pK_a values of the free substrates are approx. 6.0 (dihydroxyacetone phosphate) and 6.3 (glyceraldehyde phosphate) at 30°C, $I\ 0.1$. [These values may be compared with the widely-quoted values of Kiessling (1934) of 6.45 and 6.75 respectively.] We cannot therefore rule out the possibility that the lower pK_a values observed in the k_{cat}/K_m profiles for the isomerase-catalysed reaction arise from the ionization of the substrates. However, inspection of Fig. 4 indicates the near-identity of the pK_a values at 6.0 for reaction in the forward and reverse directions, which is expected for an ionization of the enzyme, since microscopic reversibility demands that the same groups on the enzyme are involved in both forward and reverse reactions. Further, as is discussed below, the actual pK_a of the active-site carboxyl group of triose phosphate isomerase has been shown to be about 6 (Waley, 1972).

The pH-dependences of k_{cat} are shown in Fig. 5, and arise from ionizations in enzyme-substrate complex(es). The pK_a values are 6.05 and 5.9 for reaction in the two directions, and are close to those shown by k_{cat}/K_m . If the Peller & Alberty (1959) treatment is applicable, then the enzyme group responsible for the apparent pK_a around 6.0 in the free enzyme is not significantly perturbed on substrate binding.

From the observation that after complete conversion of specifically tritiated [3- 3H]dihydroxyacetone phosphate into glyceraldehyde phosphate (and thence, by the coupling dehydrogenase, into 3-phosphoglycerate), some 2% of the 3H label has been transferred to C-2 of the product (S. G. Maister & J. Herlihy, unpublished work), it is likely that a single base on the enzyme is responsible for the shuttling of protons between C-2 and C-3 of the triose phosphates. It is conceivable that the intra-complex proton transfer does occur by the 'handing on' of the particular proton from one enzyme base to another. Even in non-enzymic reactions, intramolecular 1,3 and 1,5

proton migrations have been observed, and termed 'conducted-tour' mechanisms (Cram *et al.*, 1966). However, the assumption of a single enzymic base has the benefit of mechanistic and structural economy, and the possibility that the catalytically functional base is a carboxylate group of a glutamic acid residue (de la Mare *et al.*, 1972) would allow for an efficient proton-shuttling mechanism. Whichever substrate were presented to the enzyme, a basic carboxylate oxygen could be well placed for abstraction either of a C-2 proton from glyceraldehyde phosphate, or a C-3 proton from dihydroxyacetone phosphate. It is tempting to ascribe the lower apparent pK_a value of around 6 observed in the free enzyme to this carboxylate base. In the light of the arguments in the introduction, the strength of this conjugate acid (of pK_a above 6) would be just enough to account for the rapid rate of exchange of 3H between C-3 of dihydroxyacetone phosphate and the solvent. As pointed out above, the rate of 3H exchange with solvent demands an enzyme conjugate acid with a pK_a no higher than 6. These arguments are strengthened by the observation of Waley (1972) from modification experiments that the actual pK_a of the active-site carboxyl group in triose phosphate isomerase is about 6.

The upper pK_a value of about 9 cannot be assigned at this time. Three possible roles for an enzyme group ionizing in this region can be postulated. First, the specificity of the enzyme and the competitive inhibition shown by phosphate esters as well as by P_i , arsenate and sulphate, suggest the existence of a cationic locus at the active site that could well be provided by lysine or arginine residues. Deprotonation of a lysine residue would presumably lead to loss of substrate-binding capacity and result in the fall of k_{cat}/K_m at high pH values. Secondly, the remarkable efficiency of the proton-abstraction step, which for glyceraldehyde phosphate is faster than the turnover rate of $4700s^{-1}$ (Knowles *et al.*, 1971), suggests by analogy with aldolases of both class I and class II (Rutter, 1964) the possibility of an electrophilic catalytic component. This is most naturally formulated as a cationic group capable of hydrogen-bonding to the oxygen atom of the substrate carbonyl group. Deprotonation of such a group would again lead to a fall in k_{cat}/K_m . Thirdly, the possibility exists that the ionization at pH 9 governs a large-scale conformation change resulting in loss of enzyme activity owing to a loss of the structural integrity of the active site. Such a conformational change is believed to cause the loss of α -chymotrypsin activity observed at high pH (Sigler *et al.*, 1968). The third possibility is rendered unlikely by our observation that between pH 7 and 10, changes in the circular-dichroic spectrum for triose phosphate isomerase are negligible (see the Experimental section). The second possibility, although attractive in catalytic terms [the

enolization rate of dihydroxyacetone phosphate is only $7.9 \times 10^{-5}s^{-1}$ at $30^\circ C$ (Reynolds *et al.*, 1971): compare the k_{cat} for this substrate of $4700s^{-1}$ at $30^\circ C$], is not supported by the observation (Fig. 5) that the k_{cat} -pH profile is sigmoid, the fall in catalytic activity at high pH arises essentially entirely from a rise in K_m (Fig. 6). However, until the pH-dependence of K_i for some competitive inhibitors is known, detailed consideration of this aspect of the pH-dependences must be deferred.

Previous work on the pH-dependence of triose phosphate isomerase-catalysed reactions has been scanty. Oesper & Meyerhof (1950) reported that the catalytic activity of the calf muscle enzyme is constant between pH 7 and 8, and decreases to half its maximum value at pH 6.3. The pH-activity curves for isomerase from pea seedlings (Turner *et al.*, 1965) and from pea leaf (Anderson, 1971) are in rough agreement with this, though the algal enzyme shows a sharp optimum at pH 7.5 (Meeks *et al.*, 1968). However, Wolfenden (1970) has reported a sigmoid pH profile for k_{cat}/K_m with glyceraldehyde phosphate as substrate, showing an apparent pK_a value of 7.35. This value agreed with that obtained for the pH-dependence of the binding of the powerful competitive inhibitor 2-phosphoglycollate to the enzyme. The rabbit muscle enzyme was used in this study, and although the pH-range studied (5.5–9.0) was narrower than that in the present work, it seems unlikely that a difference of 1.3 pK units in the lower pK_a of k_{cat}/K_m could be due to the species difference between the isomerases used. No reason for this discrepancy is immediately apparent. [Dr. R. Wolfenden (personal communication) writes that kinetic constants, similar to those originally reported by him, have been obtained again with triose phosphate isomerase from rabbit muscle, but at substantially lower pH values. The results are consistent with a revised pK_a in the neighbourhood of 6.6 for the rabbit muscle enzyme in 0.05M-imidazole-HCl buffer at $25^\circ C$. The original results can only be explained by an error in the buffer used as a standard for pH determinations.]

In summary, the pH profiles for k_{cat}/K_m for the forward and reverse reactions catalysed by triose phosphate isomerase show apparent pK_a values of about 6.0 and 9.0. The k_{cat} -pH profiles are sigmoid, with apparent pK_a 6.0. The near identity of the k_{cat} profiles, and the existence of a small amount of transfer of protons from C-3 of dihydroxyacetone phosphate to C-2 of glyceraldehyde phosphate, is consistent with a single base on the enzyme being responsible for proton abstraction and proton donation to carbon centres. This would provide an effective proton-shuttling mechanism, between the C-2 and C-3 carbon atoms on one side of the substrate. The lower pK_a of 6.0 can tentatively be assigned to the enzymic base responsible for proton abstraction from carbon, probably the glutamic acid

residue identified by chemical labelling (de la Mare *et al.*, 1972; Waley, 1972), this group being fairly accessible to solvent, and having a pK_a value consistent with the upper limit set by ^3H transfer experiments.

We gratefully acknowledge the support of the Science Research Council. We are also very grateful to Miss S. de la Mare for helpful discussions. This is a contribution from the Oxford Enzyme Group.

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