APPARENT EQUIVALENCE OF THE ACTIVE-SITE GLUTAMYL RESIDUE AND THE ESSENTIAL GROUP WITH pK_a 6.0 IN TRIOSEPHOSPHATE ISOMERASE

Fred C. Hartman and Harry Ratrie III*

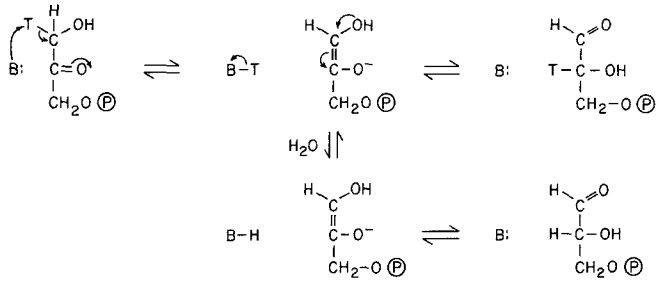
Biology Division, Oak Ridge National Laboratory,† and
The University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences,
Oak Ridge, Tennessee 37830

Received June 8,1977

SUMMARY

An anomaly has existed with respect to the catalytic group of triosephosphate isomerase that effects proton transfer: Based on kinetic studies the group has a pK_a of 6.0, whereas based on studies with affinity labels the group is a glutamyl carboxyl with pK_a of 3.9. To ascertain if these two pK_as represent two different active-site residues, we selected yeast isomerase for an evaluation of the pH-dependencies of V_{max} and K_M, since this enzyme, in contrast to muscle isomerase used in earlier kinetic work, is sufficiently stable to permit studies at the low pHs necessary to discern a catalytic group with pK_a 3.9. From pH 4.2 to 7.4 profiles of both V_{max} vs. pH and V_{max}/K_M vs. pH exhibit single ionizations that correspond to groups with pK_as of 4.6 and 5.9, respectively. Since only one group can be detected in the V_{max} profile, we conclude that it represents the essential glutamyl carboxyl and that the acidity of this carboxyl is lowered to different extents in the muscle and yeast enzymes upon substrate binding. We propose that the group with pK_a 5.9 seen in the V_{max}/K_M profile is the secondary ionization of the substrate's phosphate group.

Triosephosphate isomerase (EC 5.3.1.1) catalyzes the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. Elegant studies from the laboratories of I. A. Rose (1–3) and J. R. Knowles (4–10) have provided substantial insight into the reaction pathway:



*Predoctoral Fellow supported by Grant E (40-1) 4969 from the Energy Research and Development Administration.

†Operated by the Union Carbide Corporation for the Energy Research and Development Administration.

ISSN 0006-291X

Fundamentally the enzyme serves to shuttle protons between C1 and C2 of substrate. The conjugate acid (B–H) of the essential base (B:) ionizes (i.e. equilibrates with solvent protons) much more rapidly than it protonates the cis-enediol intermediate, so that with dihydroxyacetone phosphate stereoselectively labeled with tritium at C1, only 3–6% of the label appears in glyceraldehyde 3-phosphate (5). With this information and V_{max}, the calculated upper limit for the pK_a of the basic group is 5–6 (3, 11). In a meticulous study (11) of the pH-dependendences of V_{max} and K_M with triosephosphate isomerase from chicken breast muscle, two essential groups were observed with apparent pK_as of 6.0 and 9.0. The group with pK_a 6.0 was assumed to represent the base that shuttles protons.

From affinity labeling studies (12–16), the basic group that transfers protons is believed to be a glutamyl γ-carboxylate, corresponding to residue 165 in the rabbit muscle enzyme (17). Recent crystallographic data are consistent with this postulate (18). The essential carboxyl group in yeast triosephosphate isomerase has a pK_a of 3.9 as determined by the pH-dependence of inactivation by chloroacetol sulfate, a reagent that selectively esterifies the active-site carboxylate (19). The pK_a of the corresponding residue in muscle isomerase is not known precisely because of instability of the enzyme at low pH, but it is well below 5 (16, 19).

Thus, from kinetic studies, the essential base has a pK_a of 6.0 (in the enzyme–substrate complex), but the glutamyl residue that is thought to be the essential base has a pK_a of 3.9 (in the free enzyme). Perhaps the pK_a of the carboxyl group is perturbed upon substrate binding so that the two pK_as actually represent the same group. Alternatively, the two pK_as might represent two different essential residues, the one with pK_a 3.9 having not been detected in the earlier kinetic study, because enzyme instability precluded carrying out experiments below pH 5.4. Without a distinction between these two possibilities, uncertainty remains as to whether the active-site glutamyl residue is actually involved in proton transfer. Furthermore, if there are two essential residues with pK_as 3.9 and 6.0, the function of one of these residues is unknown, and questions arise as to the completeness of the single-base mechanism originally proposed (1). Therefore, we have examined the pH-dependences of V_{max} and K_M with yeast triosephosphate isomerase, since this enzyme can be studied over the pH range necessary to distinguish between groups with pK_as of 3.9 or 6.0.

MATERIALS AND METHODS

All solutions were prepared with deionized, glass-distilled water. Triosephosphate isomerase from yeast (Saccharomyces cerevisiae Hansen) was isolated as described earlier (15) and dialyzed against 0.02 M sodium acetate/0.1 mM EDTA (pH 6.0). The isomerase con-

centration was determined from $A_{280\,nm}$ using $E^{1\%}_{1\,cm}$ of 10.0 (15). Glycerophosphate dehydrogenase (ammonium sulfate suspension), DL-glyceraldehyde 3-phosphate diethylacetal, NADH, and Pipes‡ were obtained from Sigma Chemical Co.

The dehydrogenase was dialyzed against 0.01 M Pipes/0.1 mM EDTA (pH 6.0) containing NaCl to give a final ionic strength of 0.1. Glyceraldehyde 3-phosphate diethylacetal was converted to the free aldehyde according to the manufacturer's instructions, and the resulting stock solutions were adjusted to the same pH (with 1 N NaOH) as that of the assay medium and to an ionic strength of 0.1 with 4 M NaCl. Concentrations of D-glyceraldehyde 3-phosphate were determined enzymically with triosephosphate isomerase, glycerophosphate dehydrogenase, and excess NADH (20).

Isomerase assays were carried out at 25° with a Beckman Acta V recording spectrophotometer. This assay consists in monitoring the oxidation of NADH at 340 nm concomitant with the glycerophosphate dehydrogenase-catalyzed reduction of dihydroxyacetone phosphate formed from D-glyceraldehyde 3-phosphate (20). The buffers used in the assay were 0.1 M acetic acid/NaOH (pH 4.2-5.6) or 0.1 M Pipes/NaOH (pH 5.6-7.4). Each buffer was adjusted to an ionic strength of 0.1 with 4 M NaCl. The assay solution contained 2.0 ml of the desired buffer, 0.2 ml of 2.25 mM NADH dissolved in 0.1 M NaCl, 0.2 ml of the dialyzed glycerophosphate dehydrogenase (2.0 mg/ml), and 0.1–0.6 ml of glyceraldehyde 3-phosphate (stock concentrations of 2.5 or 25 mM in D-isomer). Sodium chloride (0–0.5 ml of 0.1 M) was added to give a final volume of 3.0 ml. The reactions were initiated by the addition of 40 ng of isomerase in 10 µl for assays at pH 4.2–4.6, 20 ng at pH 4.8–5.4, or 8 ng at pH 5.6–7.4. At every pH used, the observed initial rates were directly proportional to the isomerase concentration. The conversion of 1 µmol of substrate to product per min (one unit) gives an A_{340nm} change of 2.07.

V_{max} and K_M were obtained from least-squares analyses of Lineweaver-Burke plots constructed from the initial rate data (21). From these values, pK_as were calculated using computer programs provided by W. W. Cleland.

RESULTS AND DISCUSSION

Experimentally determined values for V_{max} and K_M in two independent series of experiments over the pH range 4.2–7.4 are given in Table 1. When log V_{max} is plotted as a function of pH, the data provide a good fit for the ionization of a single group with an apparent pK_a of 4.59 ± 0.02 (Fig. 1). The initial slope is less than 1, because the lowest pH used is so close to the pK_a of the group being titrated. Reliable assays at pH < 4.2 could not be obtained due to decomposition of NADH. A plot of log V_{max}/K_M vs. pH also shows that a single group ionizes, but with an apparent pK_a of 5.85 ± 0.02 (Fig. 1). In this case the initial slope is 1, in contrast to a slope of 2 which should be obtained if two groups were ionizing.

In an earlier study with chicken breast triosephosphate isomerase, Plaut and Knowles (11) observed ionization of a group with pK_a 6.0 in both the V_{max} and V_{max}/K_M profiles.

---

‡Abbreviation used: Pipes, 1,4-piperazinediethanesulfonic acid.

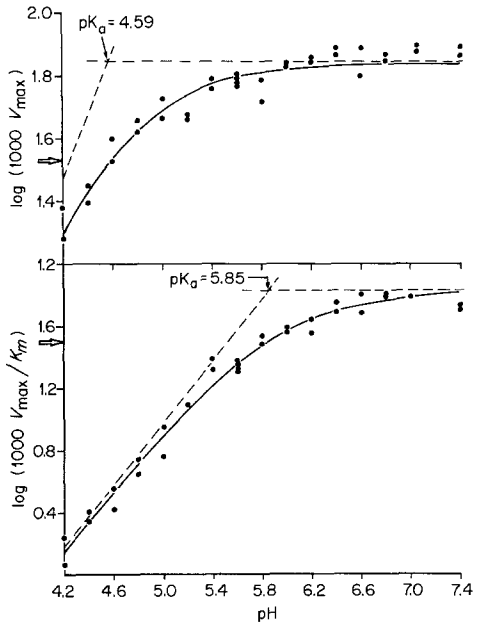


Fig. 1. Dixon-Webb plots of log V_{max} vs. pH (top) and log V_{max}/K_M vs. pH (bottom). The solid circles are actual data points, and the solid lines are computerized fits of the data to the equation log V_{max} (or log V_{max}/K_M) = log $[C/(1 + H/K_a)]$, where C is the pH-independent V_{max} or pH-independent V_{max}/K_M that would be observed when the respective ionizing groups are fully deprotonated. The computer calculated pK_as are 4.59 and 5.85 for the log V_{max} and log V_{max}/K_M curves, respectively. The computer calculated log C is 1.84 for log V_{max} and 1.81 for log V_{max}/K_M; these values are indicated by the horizontal dashed lines. The dashed lines that intersect the horizontal dashed lines at the pK_as are drawn with slopes of 1; they become tangential to the data curves at low pH, as is evident in the bottom panel. This is not obvious in the top panel, because, as indicated in the text, the pK_a (4.59) is so close to the lowest pH used (4.2) that the observed slope of the curve at low pH will be less than 1. The arrows on the ordinate mark 1/2 V_{max} (1000) and 1/2 (V_{max}/K_M) (1000).

They concluded that the group in both profiles must be the essential glutamyl γ-carboxyl and therefore that substrate binding does not alter its pK_a. However, subsequent to their study it was shown that the pK_a of the essential carboxyl in the free enzyme is only 3.9 (19). Since all ionizations observed in V_{max}/K_M profiles must represent groups in the free enzyme or free substrate, it seems unlikely that the pK_a of 6.0 in the chicken enzyme and the pK_a of 5.9 in the yeast enzyme calculated from V_{max}/K_M represent the essential glutamyl residue. An alternative possibility is that the group detected is the secondary ionization (pK_{a2}) of glyceraldehyde 3-phosphate.[1] This postulate would account for the very similar values seen

[1] This suggestion was made to the authors by W. W. Cleland.

TABLE 1

Kinetic Parameters for the Triosephosphate Isomerase-Catalyzed Reaction with D-Glyceraldehyde 3-Phosphate as Substrate

	pH	K_M (mM)	V_{max} (units/4 ng in 3-ml assay)	log 1000 V_{max}	log (1000 V_{max}/K_M)
Acetate	4.2	13.9, 16.7	0.019, 0.024	1.28, 1.38	0.06–0.24
	4.4	9.3, 10.7	0.024, 0.028	1.38, 1.45	0.35–0.41
	4.6	11.0, 12.8	0.034, 0.040	1.53, 1.60	0.42–0.56
	4.8	8.3, 9.3	0.042, 0.046	1.62, 1.66	0.65–0.74
	5.0	6.1, 8.0	0.047, 0.054	1.67, 1.73	0.77–0.95
	5.2	3.3, 3.3	0.046, 0.046	1.66, 1.66	1.14
	5.4	2.5, 2.8	0.057, 0.061	1.76, 1.79	1.31–1.39
	5.6	2.8, 2.7	0.059, 0.062	1.77, 1.79	1.31–1.35
Pipes	5.6	2.7, 2.9	0.059, 0.060	1.77, 1.78	1.32–1.36
	5.8	1.7, 1.7	0.053, 0.060	1.72, 1.78	1.49–1.54
	6.0	1.8, 1.9	0.067, 0.069	1.83, 1.84	1.57–1.58
	6.2	1.7, 2.0	0.071, 0.074	1.85, 1.87	1.55–1.64
	6.4	1.5, 1.4	0.074, 0.078	1.87, 1.89	1.69–1.75
	6.6	1.3, 1.2	0.064, 0.077	1.81, 1.89	1.69–1.81
	6.8	1.2, 1.3	0.074, 0.071	1.85, 1.86	1.74–1.79
	7.0	1.3, 1.3	0.079, 0.076	1.88, 1.90	1.77–1.78
	7.4	1.4, 1.5	0.078, 0.075	1.88, 1.89	1.70–1.75

with both the chicken and yeast enzymes. The observed pK_a is somewhat lower than the pK_{a2} of 6.3 for glyceraldehyde 3-phosphate determined directly (11), but this may be due to the slow rate of dissociation of glyceraldehyde 3-phosphate from the enzyme (4). The appearance of pK_{a2} of substrate in the V_{max}/K_M profile is consistent with the likelihood that only doubly ionized groups are bound at the site for phosphate (19). The essential carboxyl group in the free enzyme (pK_a 3.9) would presumably be detected in the V_{max}/K_M profile, if the enzyme could be assayed at lower pH.

The conclusion that the ionization with pK_a 4.6 in the V_{max} profile is the essential carboxyl seems inescapable. Only one ionization can be detected, and if only dianionic forms of substrates are bound, the secondary phosphate should not appear in the V_{max} profile. That the carboxyl group in the free enzyme has a pK_a of 3.9 does not argue against the present assignment, since the ionization in the V_{max} profile represents a group in the enzyme–substrate complex. Substrate binding could shift the pK_a of the essential carboxyl groups to 4.6 in the yeast isomerase and to 6.0 in the chicken isomerase. The degree of perturbation is of course reflective of the microenvironment within the enzyme–substrate complex and therefore is not necessarily species invariant.

The data presented show that in yeast triosephosphate isomerase a single essential group in the enzyme-substrate complex ionizes between pH 4.2 and 7.4. This result is consistent with the active-site glutamyl residue as the group that transfers protons in the isomerase catalyzed reaction.

ACKNOWLEDGMENT

The authors are grateful to Professor W. W. Cleland of the University of Wisconsin for analysis of some of the data, helpful suggestions, and critically reviewing this manuscript prior to submission.

REFERENCES

1. Rose, I. A. (1962) Brookhaven Symp. Biol. 15, 293-309.
2. Rose, I. A. (1970) in The Enzymes (Boyer, P. D., ed.), 3rd Ed., Vol. 2, pp. 281-320, Academic Press, New York.
3. Rose, I. A. (1975) in Advances in Enzymology (Meister, A., ed.), Vol. 43, pp. 491-517, John Wiley and Sons, New York.
4. Knowles, J. R., Leadlay, P. F., and Maister, S. G. (1972) Cold Spring Harbor Symp. Quant. Biol. 36, 157-164.
5. Herlihy, J. M., Maister, S. G., Albery, W. J., and Knowles, J. R. (1976) Biochemistry 15, 5601-5607.
6. Maister, S. G., Pett, C. P., Albery, W. J., and Knowles, J. R. (1976) Biochemistry 15, 5607-5612.

7. Fletcher, S. J., Herlihy, J. M., Albery, W. J., and Knowles, J. R. (1976) Biochemistry 15, 5612-5617.
8. Leadlay, P. F., Albery, W. J., and Knowles, J. R. (1976) Biochemistry 15, 5617-5620.
9. Fisher, L. M., Albery, W. J., and Knowles, J. R. (1976) Biochemistry 15, 5621-5626.
10. Albery, W. J., and Knowles, J. R. (1976) Biocehmistry 15, 5627-5631.
11. Plaut, B., and Knowles, J. R. (1972) Biochem. J. 129, 311-320.
12. Hartman, F. C. (1971) Biochemistry 10, 146-154.
13. Miller, J. C., and Waley, S. G. (1971) Biochem. J. 123, 163-170.
14. De La Mare, S., Coulson, A. F. W., Knowles, J. R., Priddle, J. D., and Offord, R. E. (1972) Biochem. J. 129, 321-331.
15. Norton, I. L., and Hartman, F. C. (1972) Biochemistry 11, 4435-4441.
16. Schray, K. J., O'Connell, E. L., and Rose, I. A. (1973) J. Biol. Chem. 248, 2214-2218.
17. Corran, P. H., and Waley, S. G. (1975) Biochem. J. 145, 335-344.
18. Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., Pogson, C. I., Wilson, I. A., Corran, P. H., Furth, A. J., Milman, J. D., Offord, R. E., Priddle, J. D., and Waley, S. G. (1975) Nature 255, 609-614.
19. Hartman, F. C., LaMuraglia, G. M., Tomozawa, Y., and Wolfenden, R. (1975) Biochemistry 14, 5274-5279.
20. Beisenherz, G. (1955) Methods Enzymol. 1, 387-391.
21. Cleland, W. W. (1967) in Advances in Enzymology (Nord, F. F., ed.), Vol. 29, pp. 1-32, John Wiley and Sons, New York.