tralization of the positive charge, e.g., [ω-NO_2-Arg[8]]GnRH, diminished the biological activity (Hazum et al., 1977). However, replacement of arginine with basic amino acids such as homoarginine, ornithine, or lysine results in analogues with substantial biological activity (Yabe et al., 1974). Taken together, these results suggest that the driving force for the formation of the hormone–receptor complex is an ionic interaction between the amino acid arginine in position 8, which is positively charged, and the carboxyl groups in the binding site.

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Enzyme Relaxation in the Reaction Catalyzed by Triosephosphate Isomerase: Detection and Kinetic Characterization of Two Unliganded Forms of the Enzyme[†]

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ABSTRACT: Triosephosphate isomerase has been shown to exist in two unliganded forms, one of which binds and isomerizes (*R*)-glyceraldehyde 3-phosphate and the other of which binds and isomerizes dihydroxyacetone 3-phosphate. The tracer perturbation method of Britton demonstrates the kinetic significance of the interconversion of these two enzyme forms at high substrate concentrations and yields a rate constant of about 10^6 s^{-1} for the interconversion. Although the molecular nature of the two forms of unliganded enzyme is not defined by these experiments, a shuffling of protons among active site residues, or a protein conformational change, or both, may be involved. This study, coupled with the known rate constants for the substrate-handling steps of triosephosphate isomerase catalysis, completes the kinetic characterization of the catalytic cycle for this enzyme.

Catalysis is a cyclic process. After facilitating the conversion of substrate to product, the catalyst must return to the form that accepts substrate so that the next cycle can begin. The regeneration of the substrate-accepting form of a catalyst may not be a trivial process, especially in enzymatic reactions where the transformation of substrate to product can involve a variety of molecular events (Walsh, 1979). At one extreme, relatively large-scale motions are believed to occur during catalysis by enzyme systems in which coenzymes are covalently bound to the protein and carry reaction intermediates from site to site. For example, in most carboxylases biotin is attached to the ε-NH_2 group of a lysine residue, and the cofactor appears to act as a mobile carboxyl group carrier between the catalytic sites of transcarboxylase (Northrop, 1969; Gerwin et al., 1969), of acetyl coenzyme A carboxylase (Guchhait et al., 1974a,b; Polakis et al., 1974), and of pyruvate carboxylase (Goodall et al., 1981). Analogously, lipoic acid residues are part of

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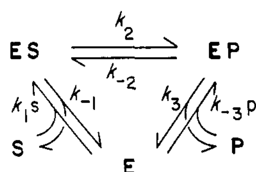
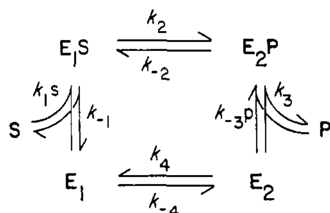
Scheme I



Scheme II



flexible domains that ferry acetyl groups among the subunits of the pyruvate dehydrogenase complex (Bates et al., 1977; Danson et al., 1978; Stephens et al., 1983). Finally, the 4'-phosphopantetheine group of the acyl carrier protein may also be a swinging arm that carries acyl groups from one enzyme to the next during fatty acid biosynthesis (Volpe & Vagelos, 1973). At the other extreme, completion of the catalytic cycle in even the simplest of enzymatic systems is likely to require adjustment of the protonation states of acidic and basic residues. For instance, proline racemase, an enzyme that catalyzes the interconversion of (*R*)- and (*S*)-proline, has been shown to exist in two unliganded forms that seem to differ *only* in the protonation states of the two active-site cysteine residues (Albery & Knowles, 1986). Also, the turnover rate of carbonic anhydrase, an enzyme that mediates the hydration of CO_2, is increased by the presence of low concentrations of buffer ions, suggesting that a proton-transfer step is important in the recreation of the substrate-accepting form of the enzyme (Tu & Silverman, 1975). Other subtle changes, such as the precise repositioning of catalytically important side chains, may also be essential in completing the catalytic cycle of some enzymes.

The regeneration of the substrate-accepting form of an enzyme can in principle occur during or after the release of product. If regeneration occurs during product release, then the minimal kinetic scheme need contain only *one* form of free (that is, unliganded) enzyme that binds either the substrate or the product, as shown in Scheme I. On the other hand, if enzyme regeneration occurs after the release of product, then *two* forms of free enzyme are required, one of which binds the substrate and the other of which binds the product, as shown in the "iso" mechanism of Scheme II (Blum, 1955; Cleland, 1963).

Enzymologists generally presume that enzyme catalysts follow Scheme I. For some systems, this presumption is improper and may well be erroneous. Indeed, the kinetic characterization of an enzyme must be considered incomplete until experiments have been performed to determine whether the system obeys Scheme I or II: that is, whether enzyme relaxation can be kinetically significant. In the present study, we have investigated the kinetic importance of multiple unliganded enzyme forms in the catalytic cycle of triosephosphate isomerase.

Triosephosphate isomerase is a glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone 3-phosphate[1] and (*R*)-glyceraldehyde 3-phosphate. Early work by Rose (Reider & Rose, 1959; Rose, 1962) defined the overall mechanism of the isomerase-catalyzed reaction (Figure 1). More recently, the rate constants for the substrate-handling steps have been determined (Albery & Knowles, 1976; Knowles & Albery, 1977). Still, we cannot claim to understand the complete energetics of the reaction catalyzed by triosephosphate isomerase without some exploration of the possible kinetic significance of the interconversion of two forms of unliganded enzyme. One of very few approaches to this problem is the tracer perturbation method of Britton (1966, 1973), in which the time-dependent distribution of radiolabeled substrate and product is measured after the system is perturbed from equilibrium by the addition of a relatively large amount of *un*labeled product. If the conversion of the "substrate" state of the free enzyme (E_1 in Scheme II) to the "product" state (E_2 in Scheme II) via k_{-4} is slower than the forward reaction of E_1 + S to E_2 + P via k_1, k_2, and k_3, then we may observe an initial flux of radiolabeled material in the *opposite direction* (from S to P) to the much larger flux of unlabeled material (from P to S). The tracer perturbation method has previously been used to probe kinetically significant enzyme isomerizations for several enzymes, as listed in Table I. We have used the tracer perturbation method to detect and characterize kinetically two unliganded forms of triosephosphate isomerase, and thereby to complete the free energy profile for the reaction catalyzed by this enzyme.

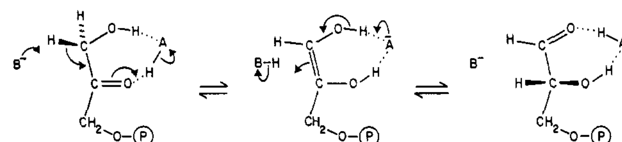


FIGURE 1: Reaction catalyzed by triosephosphate isomerase. B^- is the enzymic base (Glu-165), and HA is an enzymic acid (Lys-13 or His-95, or both).

Table I: Rates of Free Enzyme Interconversion Determined from Tracer Perturbation Experiments

enzyme	k_{cat}[a] (s^{-1})	rate of free enzyme interconversion[b] (s^{-1})	ref
phosphoglucomutase[c]	7.5×10^2	$\sim 10^7$	Britton and Clarke (1968)
phosphoglycerate mutase[d]	2.7×10^2	$>10^6$	Britton et al. (1971)
phosphoglycerate mutase[e]	1.6×10^3	$>10^6$	Britton et al. (1972)
phosphoglycerate mutase[c]	1.9×10^3	$>4 \times 10^6$	Britton and Clarke (1972)
proline racemase	2.6×10^3	$\sim 10^5$	Fisher et al. (1986b)
triosephosphate isomerase	4.3×10^3	$\sim 10^6$	this work

[a] Larger of the two values. [b] Assuming that $k_4 = k_{-4}$ in each case. [c] Rabbit muscle. [d] Wheat germ. [e] Yeast.

Experimental Procedures

Materials. Triosephosphate isomerase (chicken breast muscle) was prepared by J. G. Belasco according to Putman et al. (1972) and McVittie et al. (1972). Aldolase (rabbit muscle), fructose-6-phosphate kinase (rabbit muscle), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), hexokinase (bakers' yeast), and phosphoglucose isomerase (bakers' yeast) were obtained as crystalline suspensions in ammonium sulfate from Sigma Chemical Co. (St. Louis, MO).

[1] Nomenclature: dihydroxyacetone phosphate is dihydroxyacetone 3-phosphate; glyceraldehyde phosphate is (*R*)-glyceraldehyde 3-phosphate (or D-glyceraldehyde 3-phosphate); phosphoglycerate is 3-phospho-(*R*)-glycerate (or 3-phospho-D-glycerate).

Any triosephosphate isomerase activity in the dehydrogenase was eliminated by treatment with bromohydroxyacetone phosphate (de la Mare et al., 1972).

Bromohydroxyacetone phosphate was synthesized by the method of de la Mare et al. (1972). [1-^{14}C]Glucose (55 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). (*RS*)-Glyceraldehyde 3-phosphate (diethyl acetal, monobarium salt), Dowex 50 W (H^+ form, 100–200 mesh, 4% cross-linked), ATP[2] (disodium salt), NAD^+, and TES were from Sigma. DEAE-cellulose was from Whatman, Inc. (Clifton, NJ). Triethylamine was distilled over calcium hydride before use. All other chemicals and solvents were of commercial reagent grade or better.

Methods. Samples for radiochemical analysis were dissolved in Fisher Scinti Verse II scintillation cocktail and counted with a Beckman LS 1801 automatic liquid scintillation counter. pH measurements were made on a Radiometer RHM62 pH meter fitted with a Radiometer GK2320 C electrode, calibrated with Fisher standard buffer solutions. Fisher dialysis tubing (nominal M_r rejection 12 000–14 000) was prepared by the method of Brewer (1974). Ultraviolet absorbance measurements were made with a Perkin-Elmer 554 spectrophotometer. An extinction coefficient for NADH of 6200 M^{-1} cm^{-1} at 340 nm was assumed (Horecker & Kornberg, 1948). HPLC analyses were done on a Waters 510 instrument equipped with a Pharmacia Mono Q anion-exchange column and a Waters automated gradient controller. All operations were done at 20 °C unless otherwise noted.

[3-^{14}C]Glyceraldehyde Phosphate and [3-^{14}C]Dihydroxyacetone Phosphate. An equilibrated mixture of [3-^{14}C]-glyceraldehyde phosphate and [3-^{14}C]dihydroxyacetone phosphate was prepared by modification of the method of Yuan and Gracy (1977). A mixture (2 mL) of hexokinase (0.42 mg, 200 units), phosphoglucose isomerase (0.34 mg, 200 units), fructose-6-phosphate kinase (1.1 mg, 200 units), aldolase (15 mg, 200 units), and triosephosphate isomerase (0.019 mg, 200 units) was dialyzed exhaustively at 4 °C against 50 mM TES buffer, pH 7.5, containing $MgCl_2$ (5 mM) and KCl (10 mM). [1-^{14}C]Glucose (50 μCi, 55 mCi/mmol) was dissolved in 50 mM TES buffer, pH 7.5 (1.1 mL), containing ATP (20 μmol), $MgCl_2$ (2.5 μmol), and KCl (5 μmol). To this solution was added the dialyzed mixture of enzymes (100 μL) to give a reaction mixture containing 10 units of each enzyme. After incubation for 1.5 h, the reaction mixture was cooled to 4 °C, diluted with 25 mM triethylammonium bicarbonate buffer, pH 7.0 (20 mL), and applied to a column (11 cm × 1.7 cm^2) of DEAE-cellulose that had been equilibrated with the same buffer. The column was eluted with a linear gradient (120 mL + 120 mL) of triethylammonium bicarbonate (25–250 mM), pH 7.0, and fractions (5 mL) were collected and counted immediately (30 μL of sample in 6 mL of scintillation cocktail). Fractions containing radioactivity (fractions 18–24) were pooled, and the solution was stirred for 10 min at 4 °C with Dowex 50 W (H^+ form)(10 g wet weight). The resin was removed by filtration, and the filtrate was concentrated to approximately 3 mL by evaporation under reduced pressure. The equilibrated mixture of [^{14}C]triose phosphates (20 μCi, 28 mCi/mmol) was assayed enzymatically (see below) and stored at −70 °C.

Isomerase Reaction. A sample (3.42 μCi) of the equilibrated mixture of [^{14}C]triose phosphates was lyophilized to dryness and dissolved in 100 mM triethanolamine hydrochloride buffer, pH 7.6 (230 μL), containing EDTA (20 mM) at 30 °C. Triosephosphate isomerase (6.87 units in 10 μL of the same buffer) was then added, and the mixture was incubated at 30 °C for 15 min. Two portions (15 μL each) were removed, and each portion was quenched by injection onto a column (5 cm × 0.25 cm^2) of Dowex 50 W (H^+ form). Each column was eluted with H_2O (~6 mL), and the eluate was stored at −70 °C until analyzed.

To perturb the equilibrium, an aqueous solution (0.86 mL) of (*RS*)-glyceraldehyde 3-phosphate [784 mM, adjusted to pH 7.6 with NaOH (0.1 M) and preincubated at 30 °C] was added to the remaining 210 μL of the mixture. (Upon perturbation, the concentration of glyceraldehyde phosphate was 315 mM, the total concentration of equilibrated [3-^{14}C]-glyceraldehyde phosphate and [3-^{14}C]dihydroxyacetone phosphate was 0.1 mM, and the concentration of triosephosphate isomerase was 5.68 units/mL.) At appropriate intervals, portions (60 μL) of the reaction mixture were quenched as above, and the column eluates were stored at −70 °C until analyzed.

Concentrations of Glyceraldehyde Phosphate and of Dihydroxyacetone Phosphate. The total volume of each Dowex 50 W column eluate was determined by weight, assuming a density of 1.0 g/mL at 20 °C. The volume of the isomerization reaction quenched at each time point was determined by comparing the counts present in duplicate 100-μL portions of the Dowex 50 W column eluates with the total radioactivity present in the isomerization reaction.

The concentration of glyceraldehyde phosphate in each sample was determined by enzymatic assay with glyceraldehyde-3-phosphate dehydrogenase/NAD^+ in the presence of inorganic arsenate. The concentration of dihydroxyacetone phosphate was then determined by adding triosephosphate isomerase to the assay mixture. Each assay contained the sample (150 μL), NAD^+ (50 μL of a solution of 50 mM), Na_3AsO_4 (50 μL of a solution of 0.5 M), and 0.4 M triethanolamine hydrochloride buffer, pH 7.6 (1 mL). The absorbance at 340 nm was recorded before and 30 min after the addition of glyceraldehyde-3-phosphate dehydrogenase (10 μL of a solution of 19 mg/mL), and before and 30 min after the addition of triosephosphate isomerase (10 μL of a solution of 10 mg/mL). Duplicate assays were performed.

Distribution of ^{14}C between the Triose Phosphates. The ratio of the radioactivity in the two [^{14}C]triose phosphates was determined by first converting [3-^{14}C]glyceraldehyde phosphate to phospho[3-^{14}C]glycerate and then separating the [3-^{14}C]dihydroxyacetone phosphate from the phospho[3-^{14}C]glycerate by anion-exchange chromatography. The pH of approximately 3 mL of each Dowex 50 W column eluate was raised to 7.0 by the careful addition of NaOH (0.1 M). To this solution was added NAD^+ (200 μL of a solution of 50 mM), Na_3AsO_4 (100 μL of a solution of 0.5 M), and glyceraldehyde-3-phosphate dehydrogenase (20 μL of a solution of 19 mg/mL). The absorbance at 340 nm of a portion of the mixture was monitored in a 2-mm path length optical cuvette. When the change in absorbance with time was zero, the mixture was passed through a column (5 cm × 0.25 cm^2) of Dowex 50 W (H^+ form), and the pH of the eluate (~6 mL) was raised to 3.8 by the careful addition of aqueous triethylamine [10% (v/v)]. The resulting solution was concentrated to approximately 2 mL by lyophilization and then stored

[2] Abbreviations: ATP, adenosine 5′-triphosphate; DEAE-cellulose, (diethylaminoethyl)cellulose; DHAP, dihydroxyacetone phosphate; EDTA, ethylenediaminetetraacetate; GAP, glyceraldehyde phosphate; HPLC, high-performance liquid chromatography; NAD^+, nicotinamide adenine dinucleotide, oxidized form; NADH, nicotinamide adenine dinucleotide, reduced form; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid.

Table II: Tracer Perturbation Experiment for Triosephosphate Isomerase at 30 °C, pH 7.6

time (min)	concn of glyceraldehyde phosphate (p) (mM)	concn of dihydroxyacetone phosphate (s) (mM)	% of radioact. in glyceraldehyde phosphate $[100(p^*/c^*)]$	% of radioact. in glyceraldehyde phosphate, normalized[a] $[100(p^*/c^*)_{norm}]$	s_P (M)
0	315	0	5.1	0.2	
			5.1	0.2	
5	254	40	6.5	1.5	2.2
10	220	65	8.0	2.9	1.9
20	162	102	8.3	2.9	2.5
30	104	137	9.2	3.3	2.4
			8.8	2.9	2.6
40	67	150	9.0	2.5	2.4
50	46	164	8.3	1.3	2.9
60	31	172	7.5	0.1	
70	23	173	7.5	−0.2	
80	18	171	7.5	−0.3	
90	15	167	7.8	0.1	
100	14	166	7.4	−0.3	
110	14	166	8.1	0.3	
120	14	165	7.2	−0.6	
130	14	163	7.4	−0.4	
140	13	160	7.4	−0.4	
150	13	158	8.2	0.4	
					mean 2.4 ± 0.3

[a] The observed p^*/c^* ratio was normalized by using the p^*/c^* ratio calculated for catalysis via Scheme I: $(p^*/c^*)_{norm} = p^*/c^* - (p^*/c^*)^{I}$.

at −70 °C. About 50 nCi of this solution was injected onto an HPLC anion-exchange column, and the column was washed with 10 mM triethylammonium formate buffer, pH 3.8 (5 mL), and eluted with a linear gradient (15 mL + 15 mL) of triethylammonium formate (10–500 mM), pH 3.8. Fractions (1 mL) were collected and counted (1 mL in 10 mL of scintillation cocktail). The ratio of [3-^{14}C]dihydroxyacetone phosphate to [3-^{14}C]glyceraldehyde phosphate was determined by the ratio of the total radioactivity (corrected for background) in the fractions containing dihydroxyacetone phosphate (typically, fractions 6–8) to that in the fractions containing phosphoglycerate (typically, fractions 17–21).

Computer Simulations. The Euler–Cauchy method was used to simulate the time course of the tracer perturbation experiment. The differential equations accounted for (1) the flux of total material, (2) the flux of labeled material, (3) the hydration and dehydration of the two substrates, and (4) the nonenzymatic decomposition of the two substrates to methyl glyoxal and inorganic phosphate (Richard, 1984). The equations describing the flux of total material and the flux of labeled material were from Britton (1973). The rate constants for the substrate-handling steps were from Knowles and Albery (1977). The rate constants for hydration and dehydration of dihydroxyacetone phosphate and glyceraldehyde phosphate were from Reynolds et al. (1971) and Trentham et al. (1969), respectively, and were not corrected for the small difference in temperature. Only the unhydrated form of each substrate was presumed to bind to the enzyme (Trentham et al., 1969; Reynolds et al., 1971; Webb et al., 1977). The initial conditions and the rate constants for the nonenzymatic decomposition of dihydroxyacetone phosphate and glyceraldehyde phosphate were from the present study (see below).

Results

An equilibrated mixture of [3-^{14}C]dihydroxyacetone phosphate and [3-^{14}C]glyceraldehyde phosphate at a total triose phosphate concentration of 0.1 mM in the presence of a catalytic amount of triosephosphate isomerase was perturbed by the addition of unlabeled glyceraldehyde phosphate to a final concentration of 315 mM in (R)-glyceraldehyde-3-phosphate. The concentrations of dihydroxyacetone phosphate and glyceraldehyde phosphate, and the distribution of ^{14}C between the two triose phosphates, were then determined at timed intervals as the chemical equilibrium was reestablished. The results are presented in Table II.

The total concentration of triose phosphates ($c = s + p$, see Table II) decreases with time. The greater chemical instability of glyceraldehyde phosphate relative to dihydroxyacetone phosphate results in a more rapid loss of triose phosphate at early times (when most of the triose phosphates are present as glyceraldehyde phosphate) than later in the reaction (when the triose phosphate equilibrium has been reestablished and most of the material is present as dihydroxyacetone phosphate). The rate constants for the nonenzymatic decomposition of dihydroxyacetone phosphate and glyceraldehyde phosphate that best fit the data in Table II are 8×10^{-6} s^{-1} and 1×10^{-4} s^{-1}, respectively. These values compare well with the results of Richard (1984), who has reported 14×10^{-6} s^{-1} and 0.85×10^{-4} s^{-1}, respectively. The ratio of dihydroxyacetone phosphate to glyceraldehyde phosphate after chemical equilibrium was achieved (that is, more than 90 min after the perturbation) was 11.8 ± 0.3. The equilibrium constant therefore changes from 19 to 12 after perturbation by glyceraldehyde phosphate. This change may be due to the relative stabilization of glyceraldehyde phosphate resulting from dimer formation (Blacklow et al., 1987), but the actual reason for the establishment of a new equilibrium position does not affect the arguments below.

The time course of the fraction of the total radioactivity found in glyceraldehyde phosphate (p^*/c^*: where p^* is the concentration of [3-^{14}C]glyceraldehyde phosphate, s^* is the concentration of [3-^{14}C]dihydroxyacetone phosphate, and $c^* = s^* + p^*$) is shown in Figure 2. The ratio of [3-^{14}C]dihydroxyacetone phosphate to [3-^{14}C]glyceraldehyde phosphate after *isotopic* equilibrium was achieved (that is, more than 60 min after the perturbation) was 12.2 ± 0.5. In order to interpret the variation in p^*/c^*, we must know what the distribution of labeled material would be if Scheme I were the proper description of the isomerase-catalyzed reaction or, equivalently, if Scheme II described the reaction and k_4 and k_{-4} were very large. Under these circumstances, the time course of the change in p^*/c^* from the original equilibrium value (of 19) to the ultimate equilibrium value (of 12) can be calculated [as $(p^*/c^*)^{I}$, where the superscript "I" relates to a system obeying Scheme I]. This time course is shown in Figure 2 (for $k_4 = \infty$). The observed values of p^*/c^* can now

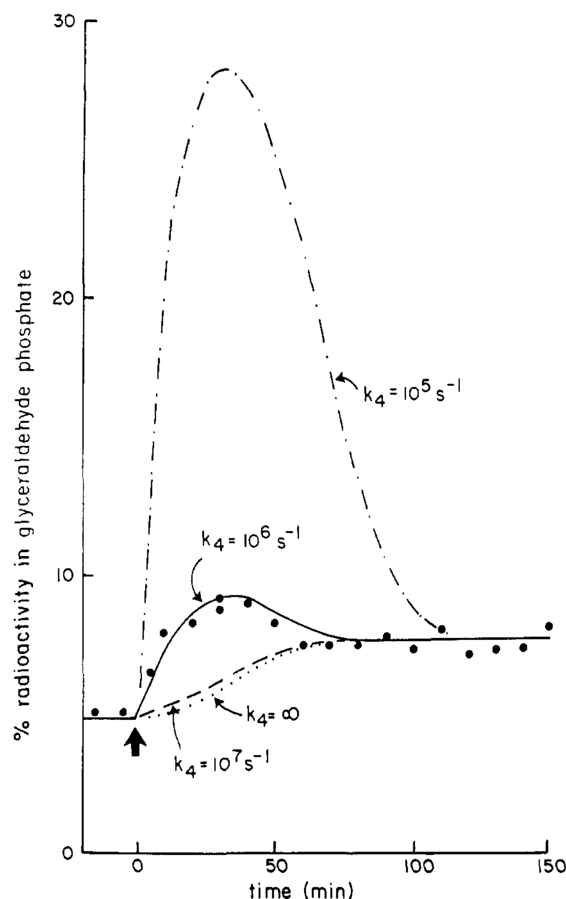


FIGURE 2: Tracer perturbation experiment for reaction catalyzed by triosephosphate isomerase: time course of the percent of the total radioactivity found in glyceraldehyde phosphate, $100(p^*/c^*)$. The lines are theoretical curves for different values of $k_4 = k_{-4}$.

be corrected by using the calculated values of $(p^*/c^*)^I$ to obtain the normalized fraction of radioactivity in glyceraldehyde phosphate $[(p^*/c^*)_{norm} = p^*/c^* - (p^*/c^*)^I]$ listed in Table II.

Discussion

The vast majority of kinetic work on enzyme-catalyzed reactions is done under "irreversible conditions": either the reaction is followed to a few percent completion in the thermodynamically downhill direction, or the concentration of product is kept at very low levels by the use of coupling enzymes. Under these circumstances, the mere determination of the steady-state parameters k_{cat} and k_{cat}/K_m cannot distinguish between enzyme systems that follow Scheme I and those that follow Scheme II. This distinction is more than merely pedantic, since—in principle at least—a k_{cat} determined under irreversible conditions may actually describe the rate of interconversion of two *unliganded* forms of the enzyme and have nothing whatever to do with the catalytic act of substrate transformation. That is, the rate-limiting step in the catalytic cycle of Scheme II could be the interconversion of E_2 and E_1. To clarify this point, we can express the steady-state parameters for the two schemes using "net" or "composite" rate constants (Cleland, 1975; Fisher et al., 1986a). A composite rate constant describes the rate of reaction via a series of intermediates and transition states and is simple to derive because the *n*th term in the reciprocal of a composite rate constant describes the free energy difference between the *n*th transition state and the original reactants. For example, the composite rate constant $k_{1,2,3}$ that describes the conversion of E + S to E + P in a system that obeys Scheme I (or, analogously, E_1 + S to E_2 + P in a system that obeys Scheme II) is defined by

$$1/k_{1,2,3} = 1/k_1 + 1/(k_2K_1) + 1/(k_3K_{1,2}) \qquad (1)$$

(where $K_{1,2} = K_1K_2$), while the composite rate constant $k_{2,3}$ that describes the conversion of ES to E + P is defined by

$$1/k_{2,3} = 1/k_2 + 1/(k_3K_2) \qquad (2)$$

By use of composite rate constants, the measured steady-state parameters (k_{cat} and k_{cat}/K_m) for Schemes I and II are

$$(k_{cat}^+/K_m^+)^I = k_{1,2,3} \qquad (3)$$

$$(k_{cat}^+/K_m^+)^{II} = k_{1,2,3}/(1 + K_4^{-1}) \qquad (4)$$

$$(k_{cat}^+)^I = (1/k_{2,3} + 1/k_3)^{-1} \qquad (5)$$

$$(k_{cat}^+)^{II} = (1/k_{2,3} + 1/k_3 + 1/k_4)^{-1} \qquad (6)$$

where k_{cat}^+ and K_m^+ relate to the forward (clockwise) direction in each scheme, and the roman numerals refer to the two schemes. From eq 3 and 4, we see that k_{cat}^+/K_m^+ for a system that obeys Scheme II is equal to that for a system which obeys Scheme I multiplied by $(1 + K_4^{-1})^{-1}$ (which is the fraction of free enzyme present at equilibrium as E_1). Further, k_{cat}^+ for a system that obeys Scheme II (eq 6) contains the rate constants both for the handling of substrate (k_2, k_{-2}, and k_3) *and* for the isomerization of the unliganded forms of the enzyme (k_4). Whether a system obeys Scheme I or II cannot be determined just from the measured values of the steady-state parameters k_{cat} and K_m because, as is clear from eq 3–6, simply knowing the *values* of these parameters does not allow us to assess the contribution to them of the rate constants for enzyme isomerization (k_4 and k_{-4}).

In 1966, Britton proposed a simple and elegant method of distinguishing a system that obeys Scheme I from one that follows Scheme II. A small quantity of radiolabeled substrate and product, S* and P*, preequilibrated in the presence of the enzyme, is perturbed by the addition of a relatively large amount of unlabeled product, P. For a system that obeys Scheme I, the ratio of the fluxes of the isotopically labeled species will not change because the increase in the concentration of the product (to $p + p^*$) that reacts with the single form of the free enzyme, E, is exactly balanced by the decrease in the specific activity of the product [to $p^*/(p + p^*)$]. The ratio of S* to P* therefore remains unchanged. On the other hand, if the system obeys Scheme II and the enzyme exists in two unliganded forms that interconvert at a rate slow enough to be kinetically significant, then, as the unlabeled product is brought to chemical equilibrium, we may observe a flux of labeled material that is in the *opposite direction* to the (much larger) flux of unlabeled material. Such "countertransport" of labeled material arises when the reaction of P to S drives E_2 to E_1, and the forward (clockwise) reaction starting with E_1 + S is competitive with the back (anticlockwise) conversion of E_1 to E_2. The appearance of countertransport is diagnostic of a system that obeys Scheme II.

The origin of countertransport lies in the perturbation of the equilibrium between E_1 and E_2. The rate of conversion of S* to P* is given by the product of the pseudo-first-order rate constant $k_{1,2,3}s^*$ and the concentration of E_1, while the rate of conversion of P* to S* is the product of $k_{-3,2,1}p^*$ and the concentration of E_2. If S* and P* are at equilibrium, and E_1 and E_2 are also at equilibrium, then the flux from S* to P* is equal to that from P* to S*. Indeed, as long as the E_1/E_2 equilibrium is maintained (or, equivalently, if the enzyme only exists in a single form, as in Scheme I), the S*/P* equilibrium is preserved. If, however, the k_4 and k_{-4} rate constants are

not large enough to maintain the E_1/E_2 equilibrium, then the addition of unlabeled P will drive the E_1/E_2 ratio toward E_1. This rise in the concentration of E_1 relative to that of E_2 results in a consequential rise in the flux of labeled material from S* to P* relative to that from P* to S*.

Although countertransport occurs whenever the E_1/E_2 equilibrium is perturbed, the phenomenon will only be observed experimentally at sufficiently high substrate concentrations. In terms of Scheme II, $k_{1,2,3}s$ must become comparable with k_{-4}. The substrate concentration at which the reaction of E_1 + S to E_2 + P is as rapid as the conversion of E_1 to E_2 is called the "peak switch concentration"[3] for the substrate (s_P) and can be written

$$s_P = k_{-4}/k_{1,2,3} \quad (7)$$

When the substrate concentration s exceeds s_P, the reaction of E_1 + S to E_2 + P occurs more rapidly than the conversion of E_1 to E_2. When s is much less than s_P, E_1 is not captured by S but has time to equilibrate with E_2.

The fact that, in the perturbation experiment with triosephosphate isomerase shown in Figure 2, countertransport of labeled substrate is clearly observed demonstrates that this enzyme exists in two kinetically significant states, E_1 and E_2, one of which binds and isomerizes glyceraldehyde phosphate, and the other of which binds and isomerizes dihydroxyacetone phosphate. The distribution of isotopic label between S* and P* as a function of the overall conversion of P to S, together with the value K_{eq}, allows us to calculate a value of s_P for each time point in the experiment. The relevant equation[4] is

$$\frac{s_P}{c/(1 + K_{eq})} = \frac{\ln (p - K_{eq}s) - \ln (p_0 - K_{eq}s_0)}{\ln [1 - (c/c^*)(p^* - K_{eq}s^*)/(p - K_{eq}s)]} \quad (8)$$

where $c = s + p$ and $c^* = s^* + p^*$. This equation is exactly equivalent to eq 22 of Britton (1973) and to the simplified eq 7 (where $K_{eq} = 1.0$) of Fisher et al. (1986b). The values of s_P calculated by using eq 8 are listed in Table II. The mean value of s_P is 2.4 ± 0.3 M, which represents the total concentration of dihydroxyacetone phosphate (unhydrated and hydrated) at which the two kinetically significant transition states that determine the reaction of E_1 back to E_2 (by the k_{-4} route and by the $k_{1,2,3}$ route) are of equal free energy.

The tracer perturbation experiment gives explicit information only about the relative free energies of the kinetically significant transition states between E_1 and E_2, while revealing nothing about the ground-state free energies of E_1 and E_2. Nevertheless, we can set limits on the relative ground-state free energies of E_1 and E_2 as follows. Knowles and Albery (1977) reported that the triosephosphate isomerase association rate constants were 4×10^8 M^{-1} s^{-1} and $\geq 10^7$ M^{-1} s^{-1} for glyceraldehyde phosphate and dihydroxyacetone phosphate, respectively. Since enzyme-substrate association rates are normally in the range of 10^6–10^8 M^{-1} s^{-1} (Hammes & Schimmel, 1970), the free energy difference between E_1 and E_2 of triosephosphate isomerase cannot be large. Therefore, K_4 cannot be very far from unity (say, $10 > K_4 > 0.1$). On this basis, $k_4 \approx k_{-4}$, and from the value of s_P and the known rate constants for the substrate-handling steps, we can calculate using eq 7 the value of k_4, the rate constant for free enzyme isomerization. First, however, we must decide on the values of $k_{1,2,3}$ and $k_{-3,2,1}$. That is, since the overall equilibrium constant, K_{eq}, changes after the perturbation, the rate constants for the substrate-handling steps must change accordingly. The value of $1/K_{eq}$ changes from 19 to 12 (equivalent to a free energy change of 0.28 kcal/mol at 30 °C), and the relative free energy of the two substrate species must also change. Fortunately, the existence of hydrated forms of each substrate does not complicate the situation because under the conditions of the present study, the two forms of each substrate (unhydrated and hydrated) interconvert much more quickly than do glyceraldehyde phosphate and dihydroxyacetone phosphate. The two extreme situations are then (a) the free energy of the dihydroxyacetone phosphate species increases by 0.28 kcal/mol and (b) the free energy of the glyceraldehyde phosphate species decreases by 0.28 kcal/mol. Adjusting $k_{1,2,3}$ and $k_{-3,2,1}$ according to these two possible interpretations[5] and assuming that K_4 is unity, we can calculate using eq 7 two values for k_4: $(1.9 \pm 0.2) \times 10^6$ s^{-1} and $(1.2 \pm 0.1) \times 10^6$ s^{-1}, where the error limits are based on the experimental error in the value of s_P. Thus, regardless of why K_{eq} changes or whether K_4 is precisely unity, it is evident that the interconversion of the unliganded forms of triosephosphate isomerase occurs with a rate constant that is close to 10^6 s^{-1}.

Although the existence of two kinetically significant unliganded forms of triosephosphate isomerase that interconvert with a rate constant of around 10^6 s^{-1} is demanded by these results, the molecular nature of the two forms of the enzyme is not defined by our experiments. Other information about the enzyme must be used to advance a useful hypothesis. The crystal structure of a triosephosphate isomerase-substrate complex has been determined to 3.5-Å resolution (Alber et al., 1981), and from this structure at least three ionizable residues appear to be directly involved in catalysis: Lys-13, His-95, and Glu-165. The carboxylate group of Glu-165 abstracts either the 1(*pro-R*)-proton of dihydroxyacetone 3-phosphate or the 2-proton of (*R*)-glyceraldehyde 3-phosphate, and this residue is unlikely to be protonated in either free enzyme form. Lys-13 and His-95 may behave as electrophiles to facilitate the enolization steps. Specifically, His-95 may be able to transfer its N_ϵ proton to the carbonyl group of glyceraldehyde phosphate. (The distance in the complex from N_ϵ of His-95 to O_1 of the substrate is ~2.6 Å.) Analogously, Lys-13 may be in a position to polarize the carbonyl group of dihydroxyacetone phosphate. (The distance from the N_ϵ of Lys-13 to O_2 of the substrate is ~3.7 Å.) The interconversion of E_1 and E_2 may therefore involve a shuffling of protons between two enzymic residues. Specifically, Lys-13 may be positively charged and His-95 electronically neutral in E_1, while His-95 would be positively charged and Lys-13 neutral in E_2. Although we cannot be sure whether a protein conformational change differentiates the two forms of unliganded enzyme (Alber et al., 1982; Karplus & McCammon, 1983; Fox et al., 1986; Elber & Karplus, 1987), it is tempting to draw a parallel with proline racemase, for which there is good evidence that the two free enzyme forms differ only in the state of protonation of the two catalytic thiol residues (Belasco et al., 1986). Genetic modification experiments are in progress to establish the roles of Lys-13 and His-95 in triosephosphate isomerase catalysis, and these results may illuminate this question.

[3] This is the substrate concentration at which the two kinetically significant transition states (the "peaks") that determine the reaction of E_1 back to E_2 (namely, that for k_{-4} and that for $k_{1,2,3}$) are of equal free energy, at which eq 7 holds (Fisher et al., 1986a).

[4] Implicit in the derivation of eq 8 is the assumption that $k_4 = k_{-4}$, which is reasonable for triosephosphate isomerase (see text).

[5] Any effects resulting from nonideal solution behavior deriving from the high substrate levels after the perturbation are unlikely to alter any of the rate constants significantly (Britton & Clark, 1968).

Must enzymatic catalysis *always* proceed by Scheme II as distinct from Scheme I? That is, does every enzyme have two unliganded forms, one of which binds the substrate(s) and the other of which binds the product(s)? Although any answer to this question must be speculative, the existence of two forms of unliganded enzyme is not surprising, because any enzyme must bind both its substrate and its product. The state of the enzyme as product dissociates is (almost) inevitably different from the state of the enzyme that will eventually bind another substrate. Still, the detection of two free forms may be problematic if the forms interconvert rapidly. For example, the tracer perturbation method of Britton (1966, 1973) will demonstrate the existence of two free forms only if $k_{1,2,3}s$ becomes measurably comparable to k_{-4}. If the maximal enzyme–substrate association rate is 10^8 M^{-1} s^{-1} (Hammes & Schimmel, 1970) and a practical upper limit on s is 1 M, then the existence of two free forms will be made apparent only if the rate constant for their interconversion is less than 10^8 s^{-1}. In some instances the tracer perturbation method has indeed failed to detect the existence of two free enzyme forms, allowing only a lower limit to be set on the rate of free enzyme interconversion (Table I). In the present case, however, the tracer perturbation method has successfully uncovered the existence of two unliganded forms of triosephosphate isomerase that interconvert with a rate constant of about 10^6 s^{-1}.

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