Triosephosphate Isomerase Catalysis Is Diffusion Controlled†

Stephen C. Blacklow, Ronald T. Raines,‡ Wendell A. Lim, Philip D. Zamore, and Jeremy R. Knowles*

Appendix: Analysis of Triose Phosphate Equilibria in Aqueous Solution by ^{31}P NMR

Wendell A. Lim, Ronald T. Raines,‡ and Jeremy R. Knowles*
Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138
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ABSTRACT: The rates of the forward and reverse reactions of triosephosphate isomerase catalyzed by the wild-type and by a sluggish mutant enzyme have been studied in the absence and the presence of several viscosogenic agents. For the mutant enzyme, the k_{cat} for which is some 10^3 times less than that for the wild-type enzyme, the value of k_{cat}/K_m with glyceraldehyde phosphate as substrate is almost unaffected by the presence of sucrose or glycerol, *even though* the concentration of the aldehyde form of the substrate is smaller because of hemiacetal formation. [The nature and relative amounts of the various forms of triose phosphate present in solution (free carbonyl forms, hydrates, dimers, hemiacetal adducts) have been evaluated by ^{31}P NMR and are presented in the Appendix.] The viscosogenic agents cause the substrate to bind more tightly to the enzyme, roughly compensating for the lower substrate concentration. With dihydroxyacetone phosphate as substrate, the values of k_{cat}/K_m for the mutant enzyme increase with the addition of viscosogenic agent, consistent with the tighter binding of substrate without (in this case) any concomitant loss due to hemiketal formation. These results for the mutant enzyme (known to be limited in rate by an enolization step in the catalytic mechanism) can be used to interpret the behavior of the wild-type enzyme. Plots of the relative values of k_{cat}/K_m for catalysis by the wild-type enzyme (normalized with the corresponding data for the mutant enzyme) against the relative viscosity have slopes close to unity, as predicted by the Stokes–Einstein equation for a cleanly diffusive process. In the presence of polymeric viscosogenic additives such as poly(ethylene glycol), polyacrylamide, or ficoll, no effect on k_{cat}/K_m is seen for the wild-type enzyme, consistent with the expectation that molecular diffusion rates are unaffected by the macroviscosity and are only slowed by the presence of smaller agents that raise the microviscosity. These results show that the reaction catalyzed by the wild-type triosephosphate isomerase is limited by the rate at which glyceraldehyde phosphate encounters, or departs from, the active site.

Diffusion is a barrier on the free energy surface of every bimolecular process. Since two molecules must collide before they can react, the rate at which the reactants diffuse together sets an upper limit on the rate at which a bimolecular transformation can occur. This same limit applies, of course, to reactions that are subject to homogeneous catalysis, including acid- and base-catalyzed processes and enzyme-catalyzed reactions. Although the rate-determining steps in reactions catalyzed by enzymes have usually been considered to be those involving the chemical interconversion of the enzyme-bound substrates, there is evidence that for many enzymatic reactions the rate-limiting transition state is diffusive (Cleland, 1975). In these cases, the transition-state free energies for the enzyme-mediated substrate–product interconversion will be lower than those for the diffusion together (or apart) of the enzyme and its substrate.

Upper limits for the second-order rate constants of bimolecular reactions in solution that are diffusion controlled have been proposed from theoretical considerations. The earliest model depicted the two reactants as spheres of homogeneous reactivity (Smoluchowski, 1917). This picture is inappropriate for enzyme-catalyzed reactions because the active site of an enzyme generally occupies only a small fraction of the surface of the protein (Nakatani & Dunford, 1979). More realistic estimates for the rate constants of a diffusion-controlled enzyme-catalyzed reaction have been made by modeling the active site as a small hemisphere on the surface of an otherwise inert spherical protein (Alberty & Hammes, 1958). Recent treatments have incorporated more detailed (and possibly more realistic) geometrical constraints (Solc & Stockmayer, 1973; Schurr & Schmitz, 1976; Samson & Deutch, 1978; Chou & Zhou, 1982). These studies generally predict that the second-order rate constant for a diffusion-limited enzyme-catalyzed reaction will be in the range of 10^8–10^{10} M^{-1} s^{-1}, this range being at the upper end of the observed rates of many enzyme–substrate and protein–ligand reactions (Hammes & Schimmel, 1970). The observed second-order rate constant can, of course, be considerably below this range, if the substrate only binds to a minor form of the enzyme or if the enzyme must find a rare form of the substrate.

To conclude merely from the absolute value of a second-order rate constant that a particular process is diffusion controlled is clearly unsatisfactory, and a direct experimental test is needed. Since the frequency at which the reacting species collide determines the rate of a diffusion-limited process, we must determine the effect of altering the collision frequency on the second-order rate constant. One approach has been to *increase* the frequency of collisions by attaching one of the reactants to a rotating disk. The rotating disk has well-defined hydrodynamics (Albery, 1975) and is a useful device for studying the effect of mass transport on the rate of a heterogeneous process. Thus chemical reagents (Root et al., 1981), enzymes (Castner & Wingard, 1984), and cells (Dunn & Ireland, 1984) have been attached to rotating disks, and the effect of rotation speed on rate has been determined. Although

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‡ Present address: Hormone Research Institute, University of California, San Francisco, CA 94143.

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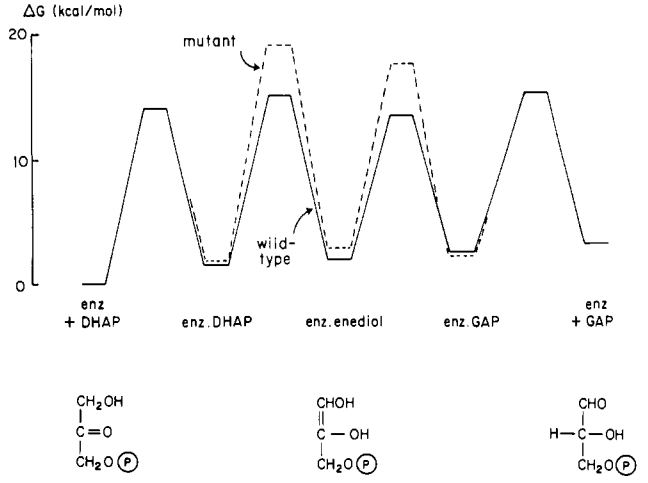

FIGURE 1: Free energy profile for the reaction catalyzed by wild-type (—) and mutant (--) triosephosphate isomerase. The wild-type profile is from Albery and Knowles (1976a). The mutant profile is from Raines et al. (1986). DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde phosphate.

this method can indeed demonstrate that the rate of a particular heterogeneous process is limited by mass transport, the technique is not capable of showing whether the corresponding homogeneous process is diffusion controlled.

The frequency with which two molecules collide is inversely proportional to the viscosity of the medium (Kramers, 1940). A straightforward way to study the effect of diffusion on the rate of a homogeneous process is therefore to *decrease* the collision frequency by increasing the viscosity of the medium. In the absence of other perturbations, the dependence of the observed second-order rate constant on viscosity can provide an estimate of the extent to which a bimolecular reaction is diffusion controlled. For example, if an enzyme-catalyzed reaction with a second-order rate constant $(k_{cat}/K_m)^0$ at viscosity η^0 is diffusion controlled, then the rate constant k_{cat}/K_m observed at a higher viscosity η will be $k_{cat}/K_m = (k_{cat}/K_m)^0(\eta^0/\eta)$. A plot of $(k_{cat}/K_m)^0/(k_{cat}/K_m)$ against η/η^0 will thus yield a straight line of unit slope when the rate of a reaction is limited by diffusion under all conditions. The technique of viscosity variation has been used to probe for diffusion control in the reactions catalyzed by phosphorylase *b* (Damjanovich et al., 1972), horseradish peroxidase (Dunford & Hewson, 1977), cytochrome *c* peroxidase (Loo & Erman, 1977), chymotrypsin (Brouwer & Kirsch, 1982), β-lactamase (Hardy & Kirsch, 1984), carbonic anhydrase (Hasinoff, 1984), invertase (Monsan & Combes, 1984), acetylcholinesterase (Bazelyansky et al., 1986), carbonic anhydrase (Pocker & Janjic, 1987), and adenosine deaminase (Kurz et al., 1987). This paper describes the use of the viscosity variation method to study the effect of diffusion on catalysis by triosephosphate isomerase, an enzyme long known to have a second-order rate constant in excess of 10^8 M^{-1} s^{-1} (Beisenherz, 1955).[1]

Triosephosphate isomerase is a glycolytic enzyme that catalyzes the interconversion of dihydroxyacetone 3-phosphate[2] and (*R*)-glyceraldehyde 3-phosphate. The mechanism of the isomerase-catalyzed reaction was originally outlined by Rose (Rieder & Rose, 1959; Rose, 1962). Subsequently, the rate constants for the individual steps were determined (Albery & Knowles, 1976a) and are depicted as a free energy profile in Figure 1. The identities of the particular ground state and the transition state that define the largest free energy difference in this profile illustrate the efficiency of triosephosphate isomerase catalysis. At the ambient substrate levels that are found in vivo, the lowest ground state in the free energy profile is not for a liganded enzyme form but relates to the unliganded enzyme plus the more stable substrate dihydroxyacetone phosphate. The highest transition state in the free energy profile involves the dissociation of the less stable substrate glyceraldehyde phosphate. If this latter step is indeed diffusive (as distinct from a step involving a conformational change in the liganded complex prior to substrate release), then the relative free energies of the highest transition state and the lowest ground state in the free energy profile are independent of the nature of the enzyme. Such an enzyme is a "perfect" catalyst, in the sense that any further acceleration of the catalytic steps [as distinct from the "on–off" steps—see Sharp et al. (1987)] would have no effect on the rate of the overall reaction (Albery & Knowles, 1976b, 1977; Knowles & Albery, 1977). The aim of this work has been to obtain experimental evidence bearing on the nature of the highest transition state in the reaction catalyzed by triosephosphate isomerase.

The reaction catalyzed by triosephosphate isomerase is well suited to the application of the technique of viscosity variation. First, since the overall equilibrium constant between dihydroxyacetone phosphate and glyceraldehyde phosphate is not very far from unity, the reaction can be studied in either direction. This capability is important, since a decrease in k_{cat}/K_m upon addition of a viscosogenic agent is not by itself sufficient evidence for the reaction being diffusion controlled. Such a decrease could, for example, derive from a decrease in the free energy of unbound substrate. Application of the Haldane equation to results obtained in both directions, however, indicates whether the free energy of one substrate has been altered relative to that of the other. Second, we know the reaction energetics of a mutant isomerase in which Glu_{165}, the essential active site base, has been replaced by Asp (Raines et al., 1986).[3] As illustrated in the free energy profile in Figure 1, catalysis by the mutant enzyme is much less efficient and is limited by chemical rather than diffusive processes. The second-order rate constant for this sluggish mutant isomerase should not vary with viscosity. The mutant enzyme therefore serves as an excellent control for any kinetically significant medium effects (other than the viscosity change) that may accompany the addition of a viscosogenic agent.

Experimental Procedures

Materials. Wild-type triosephosphate isomerase (chicken breast muscle) was prepared by J. G. Belasco according to Putman et al. (1972) and McVittie et al. (1972). Mutant (Glu_{165} to Asp) triosephosphate isomerase was prepared as described in Raines et al. (1986). Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle) and α-glycerophosphate dehydrogenase (rabbit muscle) were obtained as crystalline suspensions in ammonium sulfate from Sigma (St. Louis, MO). Each dehydrogenase was treated with bromohydroxyacetone phosphate to inactivate adventitious triosephosphate isomerase (de la Mare et al., 1972) and then dialyzed ex-

[1] Since triosephosphate isomerase only binds the unhydrated form of each substrate (Trentham et al., 1969; Reynolds et al., 1971; Webb et al., 1977), the observed values of K_m must be adjusted for substrate hydration (Albery & Knowles, 1976b).

[2] Nomenclature: dihydroxyacetone phosphate is dihydroxyacetone 3-phosphate; glyceraldehyde phosphate is (*R*)-glyceraldehyde 3-phosphate (otherwise D-glyceraldehyde 3-phosphate); glycerol phosphate is (*R*)-glycerophosphate (otherwise *sn*-glycerol 3-phosphate); phosphoglycerate is 3-phospho-(*R*)-glycerate (otherwise 3-phospho-D-glycerate).

[3] With single-letter codes for the amino acids, the mutant enzyme E165D has Glu_{165} replaced by Asp.

haustively at 4 °C against 0.1 M triethanolamine hydrochloride buffer, pH 7.6, containing EDTA[4] (10 mM) and 2-mercaptoethanol [0.01% (v/v)].

Dihydroxyacetone 3-phosphate (dimethyl ketal, cyclohexylammonium salt) was synthesized from 3-chloro-1,2-propanediol by the method of Ballou (1960). Bromohydroxyacetone phosphate was synthesized according to de la Mare et al. (1972). (*RS*)-Glyceraldehyde 3-phosphate (diethyl acetal, monobarium salt), NAD^+, NADH (disodium salt), and Dowex 50W (H^+ form, 100–200 mesh, 4% cross-linked) were from Sigma. The viscosogenic agents were ficoll (M_r 4×10^5) from Pharmacia Fine Chemicals (Piscataway, NJ), glycerol from J. T. Baker (Phillipsburg, NJ), poly(ethylene glycol) (M_r 2×10^3) from Sigma, acrylamide from Bio-Rad Laboratories (Richmond, CA), and sucrose from Bethesda Research Laboratories (Bethesda, MD). All other chemicals and solvents were of commercial reagent grade or better.

Methods. pH was measured with a Radiometer RHM62 pH meter fitted with a Radiometer GK2320 electrode, calibrated with Fisher standard buffer solutions. Ultraviolet absorbance was measured with a Perkin-Elmer 554 spectrophotometer. An extinction coefficient for NADH of 6220 M^{-1} cm^{-1} was assumed (Horecker & Kornberg, 1948).

The concentration of isomerase was based upon $A_{280}^{0.1\%}$ of 1.21 for a 10-mm light path (Miller & Waley, 1971; Furth et al., 1974). A subunit M_r of 26 500 was assumed (Putman et al., 1972; Banner et al., 1975), and kinetic parameters quoted are for a single subunit. The kinetic parameters were calculated with the nonlinear least-squares program HYPERO (Cleland, 1979). All enzyme-catalyzed reactions were run at 30 °C. All other experimental manipulations described below were done at 20 °C unless otherwise noted.

Preparation of Buffers of Various Viscosities. Each "viscosogenic buffer" contained 0.1 M triethanolamine hydrochloride buffer, pH 7.6, EDTA (5 mM), and the viscosogenic agent. The viscosogenic agents were used as obtained from the manufacturer except for polyacrylamide, which was prepared from the monomer according to the manufacturer's instructions but omitting the cross-linker, bis(acrylamide). Polyacrylamide solutions were allowed to stand overnight before use to allow for the decomposition of ammonium persulfate, which oxidizes NADH.

Measurement of Viscosity. The viscosity of each assay mixture was determined at 30 °C as the product of kinematic viscosity η/ρ, measured with an Ostwald viscometer, and density ρ. Viscosities are reported relative to 0.1 M triethanolamine hydrochloride buffer, pH 7.6, containing EDTA (5 mM).

Preparation of Triose Phosphate Solutions. Immediately before use, a solution of glyceraldehyde phosphate was prepared from the diethyl acetal by treatment with Dowex 50W (H^+ form) according to the manufacturer's instructions. The pH of the resulting solution was increased to 7.6 with NaOH (0.1 M), and the concentration of glyceraldehyde phosphate in this solution was determined by enzymatic assay (Plaut & Knowles, 1972). A solution of dihydroxyacetone phosphate at pH 7.6 was prepared from the dimethyl ketal in an analogous manner.

Glyceraldehyde Phosphate as Substrate. The initial rate of isomerization with glyceraldehyde phosphate as substrate was followed in a coupled assay with α-glycerophosphate dehydrogenase/NADH. The solution contained glyceraldehyde phosphate (30–300 μL of a solution of 10 mM), isomerase (25 μL of a solution of 1.0 unit/mL), α-glycerophosphate dehydrogenase (10 μL of a solution of 2.0 units/μL), NADH (10 μL of a solution of 50 mM), viscosogenic buffer (3.00 mL), and H_2O to a final volume of 3.40 mL. The initial decrease in absorbance at 340 nm was recorded as a function of time. The adequacy of the coupling system was tested at the highest substrate concentration used by demonstrating that the rate of the reaction was directly proportional to the concentration of isomerase.

Dihydroxyacetone Phosphate as Substrate. The initial rate of isomerization with dihydroxyacetone phosphate as substrate was followed in a coupled assay with glyceraldehyde-3-phosphate dehydrogenase/NAD^+ and inorganic arsenate. The solution contained dihydroxyacetone phosphate (20–200 μL of a solution of 40 mM), isomerase (wild type or mutant, 25 μL of a solution of 10 units/mL), glyceraldehyde-3-phosphate dehydrogenase (10 μL of a solution of 2.2 units/μL), NAD^+ (50 μL of a solution of 50 mM), Na_3AsO_4 (100 μL of a solution of 0.50 M), viscosogenic buffer (3.00 mL), and H_2O to a final volume of 3.40 mL. The initial increase in absorbance at 340 nm was recorded as a function of time. The adequacy of the coupling system was ensured as described above.

Equilibrium Constant Determinations. The equilibrium constant for dihydroxyacetone phosphate and glyceraldehyde phosphate was determined by equilibrating the substrates by use of isomerase, removing the enzyme, and assaying the concentration of each triose phosphate. A typical incubation contained dihydroxyacetone phosphate (320 μL of a 40 mM solution), isomerase (30 μL of a solution of 500 units/mL), and viscosogenic buffer (2.65 mL). The equilibrated mixture was passed through a column (5 cm × 0.25 cm^2) of Dowex 50W (H^+ form) to remove the isomerase. The pH of the eluate (~6 mL) was raised to ~7 by the addition of 0.4 M triethylammonium bicarbonate buffer, pH 7.5 (~1 mL). The concentration of dihydroxyacetone phosphate in the resulting solution was determined by enzymatic assay with α-glycerophosphate/NADH. The concentration of glyceraldehyde phosphate was then determined by addition of triosephosphate isomerase to the assay mixture.

Inhibition by Inorganic Arsenate. Triosephosphate isomerase activity was assayed with glyceraldehyde phosphate as substrate in the absence and presence of Na_3AsO_4 (14 mM) in buffer not containing any viscosogenic agent and in glycerol buffer of relative viscosity 1.7.

RESULTS

The values of k_{cat}^-/K_m^- were determined in the presence of sucrose or glycerol for the reaction of glyceraldehyde 3-phosphate catalyzed by the wild-type triosephosphate isomerase or by the mutant E165D enzyme.[3] Each value of k_{cat}^-/K_m^- is reported in Table I relative to $(k_{cat}^-/K_m^-)^0$ determined concurrently. (The "minus" superscript refers to glyceraldehyde 3-phosphate as substrate, and the "zero" superscript refers to kinetic measurements made in the absence of any viscosogenic agent when $\eta = \eta^0$.)

The steady-state kinetic parameter k_{cat}^+/K_m^+ was determined for the wild-type and mutant isomerase-catalyzed conversion of dihydroxyacetone phosphate to glyceraldehyde phosphate. (The "plus" superscript indicates that the parameter relates to dihydroxyacetone phosphate as substrate.) Each value of k_{cat}^+/K_m^+ is reported relative to the value of $(k_{cat}^+/K_m^+)^0$, in Table II. The dependence of $(k_{cat}^-/$

[4] Abbreviations: DHAP, dihydroxyacetone phosphate; EDTA, ethylenediaminetetraacetate; GAP, glyceraldehyde phosphate; NAD^+, nicotinamide adenine dinucleotide, oxidized form; NADH, nicotinamide adenine dinucleotide, reduced form.

Table I: Values of $(k_{cat}^-/K_m^-)^0/(k_{cat}^-/K_m^-)$ for the Wild-Type and Mutant Isomerases with Monomeric Viscosogenic Agents[a]

η/η^0	wild-type isomerase	mutant isomerase	η/η^0	wild-type isomerase	mutant isomerase
1.0	1.0	1.0	1.8		1.05 ± 0.08
1.3	1.2 ± 0.1		2.0	2.3 ± 0.2	
1.3	1.4 ± 0.1		2.3	2.2 ± 0.2	
1.3[b]	1.14 ± 0.07	1.03 ± 0.07	2.3[b]	2.08 ± 0.16	1.25 ± 0.10
1.4		1.09 ± 0.10	2.5[b]	2.2 ± 0.2	
1.6[b]	1.44 ± 0.14	1.04 ± 0.07	2.6	3.4 ± 0.4	1.14 ± 0.09
1.6	1.8 ± 0.3		2.9	2.7 ± 0.3	
1.7	1.8 ± 0.3		3.1		1.2 ± 0.2

[a] Glyceraldehyde phosphate is the substrate. Glycerol is the viscosogenic agent unless otherwise noted. Each k_{cat}/K_m value was determined from a minimum of six different substrate concentrations at 30 °C, pH 7.6. [b] Sucrose is the viscosogenic agent.

Table II: Values of $(k_{cat}^+/K_m^+)^0/(k_{cat}^+/K_m^+)$ for the Wild-Type and Mutant Isomerases with Glycerol as Viscosogenic Agent[a]

η/η^0	wild-type isomerase	mutant isomerase	η/η^0	wild-type isomerase	mutant isomerase
1.0	1.0	1.0	1.8		0.64 ± 0.07
1.3	1.1 ± 0.1		2.3	1.5 ± 0.2	
1.4		0.96 ± 0.08	2.6		0.47 ± 0.08
1.6		0.77 ± 0.06	2.9	1.7 ± 0.2	
1.7	1.4 ± 0.1				

[a] Dihydroxyacetone phosphate is the substrate. Each k_{cat}/K_m value was determined from a minimum of six different substrate concentrations, at 30 °C, pH 7.6.

Table III: Values of $(k_{cat}^-)^0/(k_{cat}^-)$ for the Mutant Isomerase with Sucrose or Glycerol as Viscosogenic Agent[a]

viscosogen	η/η^0	$(k_{cat}^-)^0/(k_{cat}^-)$	viscosogen	η/η^0	$(k_{cat}^-)^0/(k_{cat}^-)$
none	1.0	1.0	glycerol	1.42	1.11 ± 0.04
sucrose	1.3	1.04 ± 0.03	glycerol	1.82	1.26 ± 0.05
sucrose	1.6	1.07 ± 0.03	glycerol	2.56	1.18 ± 0.05
sucrose	2.29	1.07 ± 0.03			

[a] Glyceraldehyde phosphate is the substrate. The absolute value of $(k_{cat}^-)^0$ was found to be 3.08 ± 0.05 s^{-1}.

Table IV: Values of $(k_{cat}^+)^0/(k_{cat}^+)$ for the Mutant Isomerase with Glycerol as Viscosogenic Agent[a]

η/η^0	$(k_{cat}^+)^0/(k_{cat}^+)$	η/η^0	$(k_{cat}^+)^0/(k_{cat}^+)$
1.0	1.0	1.82	1.17 ± 0.07
1.42	1.02 ± 0.04	2.58	1.28 ± 0.04

[a] Dihydroxyacetone phosphate is the substrate. The absolute value for $(k_{cat}^+)^0$ was found to be 2.67 ± 0.09 s^{-1}.

$K_m^-)^0/(k_{cat}^-/K_m^-)$ on η/η^0 for polymeric viscosogenic additives [ficoll, polyacrylamide, and poly(ethylene glycol)] is plotted in Figure 2.

The values of k_{cat}^- in the presence of various concentrations of sucrose or glycerol were determined for the mutant E165D enzyme and are listed in Table III. The values of k_{cat}^+ determined for the mutant E165D enzyme in the presence of various concentrations of glycerol are listed in Table IV.

The equilibrium ratio of dihydroxyacetone phosphate to glyceraldehyde phosphate was measured in buffers containing poly(ethylene glycol), 2-propanol, or various concentrations of glycerol. The measured equilibrium constants are listed in Table V. Equilibrium constants can be calculated from the results of Tables I and II by application of the Haldane equation and by use of the observed value of 20.4 for the ratio $(k_{cat}^-/K_m^-)^0/(k_{cat}^+/K_m^+)^0$. These equilibrium constants are also listed in Table V.

Inorganic arsenate is a competitive inhibitor of triosephosphate isomerase. The value of K_i determined in glycerol buffer of relative viscosity 1.7 relative to the value of K_i^0 was 1.0 ± 0.1.

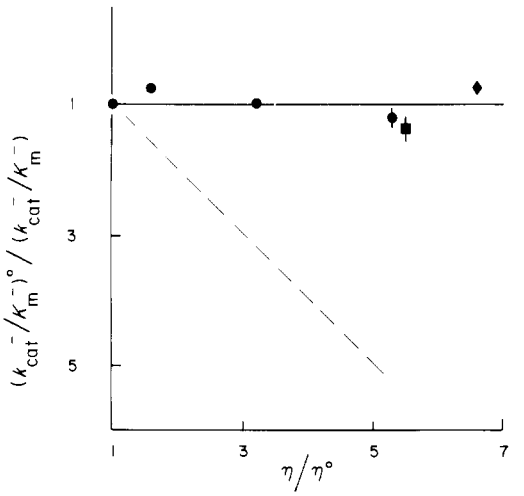

FIGURE 2: Plot of the reciprocal of the relative second-order rate constant as a function of relative viscosity when polymeric species are the viscosogenic agents and glyceraldehyde phosphate is the substrate. The viscosogenic agents used were poly(ethylene glycol) (●), polyacrylamide (■), and ficoll (♦). The dashed line has a slope of 1.

Table V: Equilibrium Constant for the Isomerase-Catalyzed Reaction in Buffers of Different Viscosity and Dielectric Constant[a]

additive	η/η^0	ϵ/ϵ^0	$1/K_{eq}$[b]	$1/K_{eq}$[c]
none	1.0	1.0	22 ± 2	20
glycerol (9%)	1.3	0.97	20 ± 1	19
glycerol (18%)	1.7	0.93	14 ± 1	16
glycerol (27%)	2.3	0.90	11 ± 1	14
glycerol (34%)[d]	2.6	0.88		14
glycerol (36%)	2.9	0.87	10 ± 1	12
poly(ethylene glycol) (18%)	3.2		19 ± 1	
2-propanol (14%)		0.86	21 ± 2	

[a] The concentrations of additive are in percent (w/w). The values of the relative dielectric constant, ϵ/ϵ^0, are estimated at 30 °C from the data in Franks (1973). Glyceraldehyde phosphate was used as the substrate for the equilibration. [b] By direct measurement. [c] From the Haldane relationship: $K_{eq} = (k_{cat}^-/K_m^-)/(k_{cat}^+/K_m^+)$. The data in Tables I and II were used. [d] Using the mutant E165D enzyme.

Discussion

Theoretical approaches have generally predicted that the second-order rate constant for a diffusion-limited enzyme-catalyzed reaction will be in the range of 10^8–10^{10} M^{-1} s^{-1} (Solc & Stockmayer, 1973; Schurr & Schmitz, 1976; Samson & Deutch, 1978; Chou & Zhou, 1982), yet this range is higher than the observed values of k_{cat}/K_m for most of the fastest enzymatic reactions (Hammes & Schimmel, 1970). There may be several reasons for this apparent discrepancy, for, unlike the diffusion of two unsolvated small molecules in solution, the binding of a substrate into the active site of its enzyme involves considerably more than a simple encounter. Substrate binding into an active site will involve changes in ion pairing, in hydrogen-bonding partners, and in local solvent structure both for the substrate and for the active site, and these exchanges will inevitably produce an enthalpic contribution to what, for small unsolvated species, is normally considered to be a purely entropic diffusion phenomenon. An enthalpic contribution to the activation free energy of the docking process will result in a slower observed rate of association. Additionally, if the substrate only binds to a rare conformational or solvation state of the enzyme's active site, then lower values for the second-order rate constant for enzyme–substrate association will be observed. Conversely, the

possibility that the enzyme is designed to exploit the Debye effect for charged substrates, and create a high electric field gradient in the neighborhood of the active site, can yield association rate constants that are higher than those predicted on the basis of mere diffusion (Sharp et al., 1987). For these reasons, we must be careful not to stretch the meaning of the phrase "diffusion control", when describing an enzymatic reaction having a rate that is limited by the "on" rate of one of its substrates.

The simplest way to establish whether or not a reaction in solution is limited by diffusion is to vary the viscosity. In this paper, we report the application of the viscosity variation method to the reaction catalyzed by triosephosphate isomerase, an enzyme that has a value of k_{cat}/K_m in the thermodynamically downhill direction of 4×10^8 M^{-1} s^{-1} (Albery & Knowles, 1976a) and seems a likely candidate for a reaction that proceeds at the diffusion limit. If the rate of a bimolecular reaction is cleanly limited by the frequency of encounter of the two reactants and if there are no effects on the reaction rate other than those deriving from the change in viscosity, the slope of a plot of the relative second-order rate constant $(k_{cat}/K_m)^0/(k_{cat}/K_m)$ versus the relative viscosity η/η^0 will be 1.0 (Kramers, 1940).

There are two classes of additive that markedly change the viscosity of aqueous solutions: (a) monomeric polyhydroxylated molecules such as sucrose or glycerol and (b) polymeric species such as poly(ethylene glycol), polyacrylamide, or ficoll. In general, the addition of polymeric viscosogenic agents affects the measured viscosity of the solution but has no effect on the diffusion behavior of small molecules or (therefore) on the rates of diffusion-controlled reactions. In contrast, the addition of small-molecule viscosogens affects both the measured viscosity *and* the rates of diffusive processes. Although this difference between the effects of monomeric and polymeric viscosogenic agents might seem surprising, an insensitivity of the second-order rate constant of a diffusion-limited reaction to the presence of the polymeric materials is predicted and reflects the important (and sometimes overlooked) difference between "microviscosity" and "macroviscosity".

Macroviscosity is a bulk property and refers to the viscosity as measured by conventional methods such as a viscometer. In contrast, microviscosity refers to the resistance to motion that a molecule experiences in solution. The distinction between macroviscosity and microviscosity was demonstrated dramatically over a century ago by Graham (1862), who showed that small ions diffuse through gelatin at the same rate that they diffuse through water. Polymeric species increase the macroviscosity of a solution but do not significantly change the rate at which small molecules diffuse (Nishijima & Oster, 1956; Biancheria & Kegeles, 1957; Stokes & Weeks, 1964; Morawetz, 1975; Phillips et al., 1977). Viscosogenic materials in this class merely reduce the free volume available to the diffusing species. Most enzymes are much larger than the substrates upon which they act, and the frequency at which such enzymes encounter their substrates depends almost completely on the rate at which the substrate diffuses. This rate is determined by the microviscosity of the solution (Welch et al., 1983; Whittenburg, 1983). Although polymers increase the macroviscosity of aqueous solutions without affecting the microviscosity, monomeric species such as sucrose and glycerol affect both the macroviscosity and the microviscosity of aqueous solutions. Polymers are clearly inappropriate viscosogenic agents to probe for diffusion control, and the values of the kinetic parameters obtained in the presence of such materials provide no information on questions concerning the diffusion limit. Such conclusions parallel the recent observations of Pocker and Janjic (1987) and Kurz et al. (1987), who note the absence of a rate decrease when using the polymer ficoll to probe diffusion-limited reactions of carbonic anhydrase and adenosine deaminase, respectively, but this point may not always have been fully recognized (Damjanovich et al., 1972; Brouwer & Kirsch, 1982; Monsan & Combes, 1984).

Although the method of viscosity variation can be a useful probe for diffusion control in enzymatic reactions, varying the viscosity of an aqueous solution without inadvertantly perturbing other parameters of the system seems to be impossible, and control experiments must be performed to ensure that the observed effects are due *only* to the viscosity change. Kirsch and his collaborators have neatly exploited the possibility of using *poor substrates* to provide the needed control in their study of hydrolytic enzymes (Brouwer & Kirsch, 1982; Hardy & Kirsch, 1984; Baselyansky et al., 1986). That is, if a good substrate reacts at or near the diffusion limit and the reaction is sensitive to solution viscosity, a poor substrate (for which the rate of reaction is determined by some step other than a diffusive one) can be used to check that the viscosogenic agent does not have other extraneous effects on the enzyme-catalyzed reaction rate. The extremely narrow substrate specificity that is typical of most intracellular enzymes, however, precludes this "poor substrate" approach with such enzymes as triosephosphate isomerase. We have, instead, used a "poor enzyme" (a sluggish mutant) as the control in the present work. From the free energy profile of the mutant isomerase in which the active site base Glu_{165} has been changed to Asp (Figure 1), we see that neither of the diffusive transition states (that is, those representing the binding of substrate or of product) is kinetically significant for the mutant enzyme, and in the absence of other perturbing effects, k_{cat}/K_m in either direction should be independent of viscosity.

The first control on the validity of the methods used is shown in Figure 2, which illustrates the invariance of the values of k_{cat}^-/K_m^- (that is, the second-order rate constant with glyceraldehyde phosphate as the substrate) for the wild-type enzyme in the presence of any of the polymeric additives, poly(ethylene glycol), polyacrylamide, or ficoll. The kinetic behavior of the enzyme is evidently unaffected by changes in the macroviscosity up to a relative viscosity of 6.6 (Figure 2). In contrast, the raw data presented in Tables I and II show that the kinetic parameters for catalysis by the wild-type enzyme are strongly dependent on the *micro*viscosity, as modulated by the addition of either of the small monomeric viscosogenic agents, glycerol or sucrose.

Now, it has been known for many years that the two substrates of this enzyme, glyceraldehyde phosphate and dihydroxyacetone phosphate, are substantially hydrated in aqueous solution and that only the unhydrated forms bind to and are substrates for the isomerase (Trentham et al., 1969; Reynolds et al., 1971; Webb et al., 1977). The measured second-order rate constant for reaction with the enzyme therefore depends on the concentration of unhydrated glyceraldehyde phosphate (or unhydrated dihydroxyacetone phosphate) in solution. To evaluate the effect of added glycerol on the proportion of unhydrated triose phosphate in solution, we have used ^{31}P NMR and have demonstrated the formation of a new species of glyceraldehyde phosphate in aqueous glycerol: the glycerol hemiacetal. These experiments are described in the Appendix, from which it is clear that the proportion of glyceraldehyde phosphate in the aldehyde form decreases as the concentration of glycerol rises. Why, then,

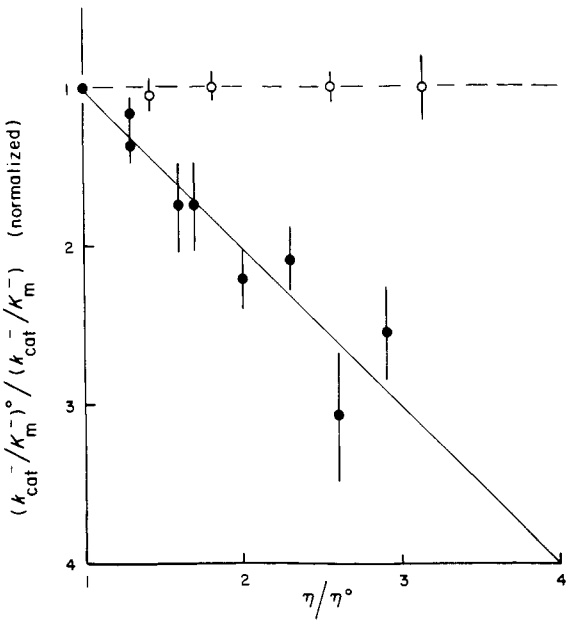

FIGURE 3: Plot of the normalized values for the reciprocal of the relative second-order rate constant for wild-type (●) and E165D mutant (○) isomerases with glyceraldehyde phosphate as substrate and glycerol as viscosogenic agent. The slope (0.09) of the least-squares regression line for the variation of $[(k_{cat}/K_m)^0/(k_{cat}/K_m)]$ with (η/η^0) for the mutant isomerase was used to normalize each value of $[(k_{cat}/K_m)^0/(k_{cat}/K_m)]$, with the expression $[(k_{cat}/K_m)^0/(k_{cat}/K_m)]_{norm} = [(k_{cat}/K_m)^0/(k_{cat}/K_m)]_{obsd} - 0.09[(\eta/\eta^0) - 1]$. The primary data were taken from Table I. The slope of the regression line for the normalized results with the mutant E165D isomerase is 0.00 ± 0.02 and with the wild-type enzyme is 1.0 ± 0.2.

is the value of k_{cat}^-/K_m^- for the mutant enzyme essentially unaffected by added sucrose or added glycerol (Table I) when we know that the concentration of the substrate form of glyceraldehyde phosphate is lower in the presence of these additives? There are only two possibilities. Either the effect is on the *ground state*, and there is a destabilization of free (versus bound) substrate that roughly compensates for the lower concentration of the aldehyde form, or there is a stabilization of the rate-limiting *transition state* for the catalyzed reaction that approximately compensates for the lower aldehyde levels. The results in Table III show that the second possibility is incorrect, since the values of k_{cat}^- for the mutant enzyme are unaffected by added sucrose or glycerol. That is, neither sucrose nor glycerol perturbs the relative free energies of the enzyme-bound states, and their effects must therefore be to lower the K_m value. In the presence of the hydroxylic viscosogenic agent, the lower concentration of the aldehyde form of glyceraldehyde phosphate is offset by its tighter binding to the enzyme. The near absence of an effect of viscosogenic agents on k_{cat}^-/K_m^- for the mutant isomerase therefore results from the cancellation of the decreased concentration of unhydrated glyceraldehyde phosphate by a decrease in the stability of unhydrated glyceraldehyde phosphate with respect to that of the enzyme-bound form. This destabilization presumably derives from changes in the solvation of the unliganded species when viscosogenic agents are present. On this basis, then, we can normalize the experimental values of k_{cat}^-/K_m^- for the wild-type enzyme and make the (small) correction for the slight change in the values of k_{cat}^-/K_m^- with viscosity for the mutant enzyme. This correction results in Figure 3, from which it is clear that, normalized against the results for mutant enzyme, the wild-type isomerase gratifyingly obeys the Stokes–Einstein prediction for a cleanly diffusion-limited reaction. The gradient of the relative second-order rate constant *versus* relative viscosity is 1.0 ± 0.2.

Unlike all previous work on viscosity variation in enzymology, we can, in the case of triosephosphate isomerase, conveniently study the reaction in the reverse direction. Following the logic used above in the discussion of the reaction of glyceraldehyde phosphate with the enzyme, we see from Table II that when dihydroxyacetone phosphate is the substrate, the values of k_{cat}^+/K_m^+ for the mutant enzyme actually *increase* with increasing levels of glycerol. Such a rate acceleration is not unprecedented (Bazelyansky et al., 1986) and in the present case is entirely consistent with the above discussion. Dihydroxyacetone phosphate does not form detectable levels of hemiketal with glycerol (see Appendix), and the concentration of the substrate keto form is unaffected by the presence of viscosogens. [The fact that glycerol forms a hemiacetal with glyceraldehyde phosphate and not a hemiketal with dihydroxyacetone phosphate is independently confirmed by the good agreement between the overall equilibrium constant determined by direct measurement and that deduced from the Haldane relationship, $K_{eq} = (k_{cat}^-/K_m^-)/(k_{cat}^+/K_m^+)$ (see Table V).[5]] Yet, even though glycerol and sucrose do not lower the concentration of the keto form of dihydroxyacetone phosphate, it *is* expected that the destabilization of free triosephosphate observed for glyceraldehyde phosphate would also be seen for dihydroxyacetone phosphate.[6] That is, we expect to decrease the stability of *both* triosephosphates with respect to their bound forms by adding viscosogens, and this, in the case of dihydroxyacetone phosphate, will lead to a *faster* second-order reaction with the mutant enzyme. This is what is observed (Table II). With the mutant enzyme, k_{cat}^+ is also unaffected, again ruling out any effect of the viscosogens on the enzyme-bound states (Table IV). Now, as before, we can use the values of k_{cat}^+/K_m^+ for the mutant enzyme to normalize the corresponding data for the wild-type enzyme and obtain Figure 4. From this figure, in which the gradient of the line is 1.0 ± 0.1, it is clear that the reaction of dihydroxyacetone phosphate catalyzed by the wild-type enzyme is limited by diffusion, quantitatively as predicted by the Stokes–Einstein relationship.[7]

It has been proposed that the rate-determining transition state in the reaction catalyzed by triosephosphate isomerase is that which involves binding of glyceraldehyde phosphate to

[5] Inorganic arsenate, a necessary component in the assay solution when dihydroxyacetone phosphate is the substrate, is a competitive inhibitor of triosephosphate isomerase (Burton & Waley, 1968). If the inorganic arsenate were to become a less effective inhibitor in the presence of high glycerol concentrations, the observed decrease in the second-order rate constant upon glycerol addition would be less marked. Since the transient formation of labile arsenate esters of sugars has precedent (Cohn, 1961; Lagunas, 1970), we investigated this point experimentally and found that the K_i for inorganic arsenate changes insignificantly when glycerol is added to the solution. This confirms the results in Table V, which show that the kinetically derived K_{eq} is in good agreement with that determined directly.

[6] One factor that is likely to contribute to this indiscriminate destabilization could be the low dielectric constant of glycerol (Franks, 1973). Since both triose phosphates are dianions at pH 7.6 (McElroy & Glass, 1951), lowering the dielectric constant of the solution will decrease the stability of the triose phosphates relative to their bound states without affecting the overall equilibrium constant. We have checked this point by measuring the overall equilibrium constant in buffer containing 14% (w/w) 2-propanol, which has a dielectric constant similar to that of the buffer containing 36% glycerol (Franks, 1973). As shown in Table V, a decrease in the dielectric constant alone does not change the overall equilibrium constant.

[7] It must, of course, be true that if k_{cat}^-/K_m^- is limited by a diffusive step, so is k_{cat}^+/K_m^+. Nevertheless, the internal consistency represented by Figures 3 and 4 provides a nice check on the precision of the kinetic experiments.

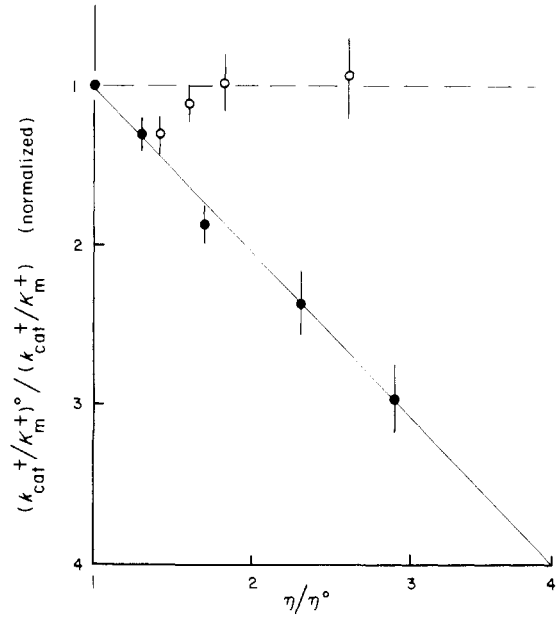

FIGURE 4: Plot of the normalized values for the reciprocal of the relative second-order rate constant for wild-type (●) and E165D mutant (○) isomerases with dihydroxyacetone phosphate as substrate and glycerol as viscosogenic agent. The slope (0.66) of the fitted least-squares regression line for the variation of $[(k_{cat}/K_m)/(k_{cat}/K_m)^0]$ with (η/η^0) for the mutant isomerase was used to normalize each value of $[(k_{cat}/K_m)^0/(k_{cat}/K_m)]$. For the mutant isomerase, the expression $[(k_{cat}/K_m)/(k_{cat}/K_m)^0]_{norm} = [(k_{cat}/K_m)/(k_{cat}/K_m)^0]_{obsd} - 0.66[(\eta/\eta^0) - 1]$ was used to derive the normalized values of $[(k_{cat}/K_m)^0/(k_{cat}/K_m)]$. For wild-type isomerase, the expression $[(k_{cat}/K_m)^0/(k_{cat}/K_m)]_{norm} = [(k_{cat}/K_m)^0/(k_{cat}/K_m)]_{obsd} + 0.66[(\eta/\eta^0) - 1]$ was used. The primary data were taken from Table II. The slope of the regression line for the normalized results with the mutant enzyme is 0.0 ± 0.2 and with the wild-type enzyme is 1.0 ± 0.1.

the enzyme (Albery & Knowles, 1976b). The results presented here confirm this assertion, which was originally based simply on the size of the second-order rate constant in the thermodynamically downhill direction for the catalyzed reaction (k_{cat}^{-}/K_m^{-}). The formation of the Michaelis complex between the enzyme and glyceraldehyde phosphate is evidently limited in rate by the frequency of encounter of the two species and not by some slower protein conformational change. Our findings further suggest that great care must be taken when the viscosity variation method is used as a probe for diffusion control: a perturbation of the original system due to added viscosogen may disturb more than just the viscosity of the solution, and such other effects must be evaluated in any interpretation of experiments in which the viscosity variation method is used. The present case of triosephosphate isomerase appears to be well behaved, and the results reported in this paper confirm that, accepting the constraints of the in vivo levels of its substrates and assuming freely diffusing substrate species, triosephosphate isomerase can improve no further as a catalyst.

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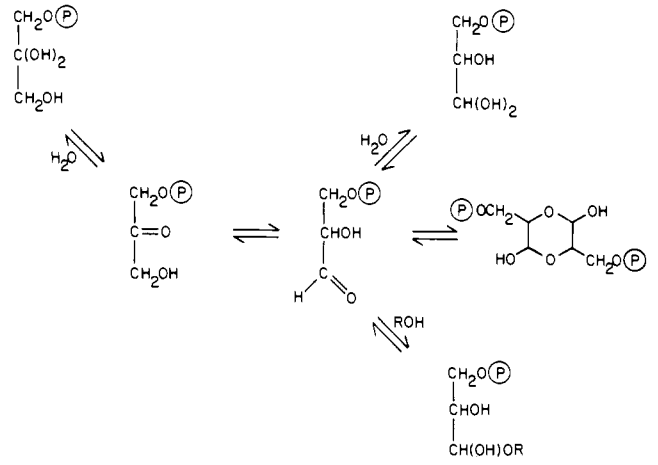
Appendix: Analysis of Triose Phosphate Equilibria in Aqueous Solution by ^{31}P NMR

The steady-state kinetic parameters of enzymes are normally determined from the dependence of reaction velocity on total substrate concentration. Glyceraldehyde phosphate (GAP) and dihydroxyacetone phosphate (DHAP), the substrates for triosephosphate isomerase (TIM), exist in aqueous solution as complex mixtures. For example, glyceraldehyde phosphate is largely hydrated in aqueous solution (Trentham et al., 1969), and can—at least in principle—form both a six-membered ring dimer and hemiacetal adducts with solution nucleophiles (Scheme I). Dihydroxyacetone phosphate can form analogous

Scheme I



species. Nevertheless, only the free carbonyl forms of glyceraldehyde phosphate and of dihydroxyacetone phosphate are substrates for triosephosphate isomerase (Trentham et al., 1969; Reynolds et al., 1971). The overall enzyme-catalyzed reaction therefore involves three equilibria (eq 1): (a) the equilibrium between the two "active" substrates, K_{TIM}, (b) the equilibrium among glyceraldehyde phosphate free aldehyde and the "inactive" species which it reversibly forms, K_{GAP}, and (c) the equilibrium among dihydroxyacetone phosphate free ketone and the "inactive" species which it reversibly forms, K_{DHAP}. Changing the solvent can perturb the equilibria of eq 1 both by differential solvation effects and through the formation of new inactive species, and such perturbations will alter the observed kinetic parameters for the enzyme-catalyzed reaction. The dependence of the overall equilibrium constant K_{eq} on the individual equilibrium constants of eq 1 is expressed by

$$DHAP_{inactive} \overset{K_{DHAP}}{\rightleftharpoons} DHAP_{active} \overset{K_{TIM}}{\rightleftharpoons} GAP_{active} \overset{K_{GAP}}{\rightleftharpoons} GAP_{inactive} \quad (1)$$

$$K_{eq} = [(1 + K_{GAP})/K_{GAP}][K_{DHAP}/(1 + K_{DHAP})]K_{TIM} \quad (2)$$

where

$$K_{eq} = [GAP_{total}]/[DHAP_{total}] \quad (3)$$

and

$$K_{DHAP} = [DHAP_{active}]/[DHAP_{inactive}]$$

$$K_{GAP} = [GAP_{active}]/[GAP_{inactive}]$$

$$K_{TIM} = [GAP_{active}]/[DHAP_{active}]$$

Since the Haldane equation defines $K_{eq} = (k_{cat}^{+}/K_m^{+})/(k_{cat}^{-}/K_m^{-})$, in any experiment in which the rate of the enzyme-catalyzed reaction is studied in a different solvent, we must characterize any changes in the equilibria of the substrates in order that changes in the kinetics of the enzymatic reaction be properly interpreted. The present study was undertaken to provide the information necessary to interpret the kinetic data from experiments on the rate of the isomerase-catalyzed reaction in the presence of viscosogenic agents (see main paper).

If glycerol is added as a viscosogenic agent to TIM kinetic assay buffers, changes in K_{GAP} and K_{DHAP} can occur because the hydroxyl groups of glycerol can potentially form hemiacetals or hemiketals with the carbonyl forms of the triose phosphates. We have used ^{31}P NMR spectroscopy to probe such changes. The peak assignments can be made as follows. First, triosephosphate adducts with solvent components can be identified by the disappearance of resonances upon removal of individual components from the solution. Second, dimeric species can be identified by the concentration dependence of the intensities of particular resonances. Third, the known extents of hydration of the triose phosphates (Trentham et al., 1969; Reynolds et al., 1971) provide information about the relative peak intensities for the resonances deriving from the hydrates and the free carbonyl forms. Finally, on the basis that the dehydration rate constants for the triose phosphate hydrates are known to be on the order of 0.5–0.01 s^{-1} (Trentham et al., 1969; Reynolds et al., 1971), we may expect to observe magnetization transfer between the hydrated and unhydrated species in an inversion transfer experiment (Alger & Schulman, 1984). In this appendix we report the identification of all stable unbound triose phosphate species and the measurement of K_{GAP} and K_{DHAP} in a range of buffers containing different concentrations of the viscosogenic agent, glycerol.

Experimental Procedures

Materials. All materials were obtained as in the main paper. Racemic glyceraldehyde 3-phosphate was used since the equilibria not involving enzyme are not enantiomer dependent. Each NMR sample at pH 7.6 contained triose phosphate (20–40 mM) and EDTA (5 mM). When present, triethanolamine was at a concentration of 0.1 M, as in the standard TIM kinetic assays (see main paper).