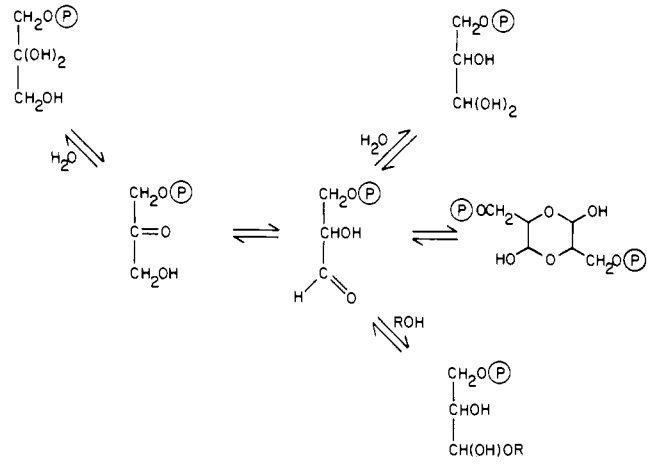
Root, K. S., Deutch, J., & Whitesides, G. M. (1981) *J. Am. Chem. Soc. 103*, 5475–5479.
Rose, I. A. (1962) *Brookhaven Symp. Biol. 15*, 293–309.
Samson, R., & Deutch, J. M. (1978) *J. Chem. Phys. 68*, 285–290.
Schurr, J. H., & Schmitz, J. (1976) *J. Phys. Chem. 80*, 1934–1936.
Segel, I. H. (1975) *Enzyme Kinetics*, pp 100–160, Wiley, New York.
Sharp, K., Fine, R., & Honig, B. (1987) *Science (Washington, D.C.) 236*, 1460–1463.
Smoluchowski, M. von (1917) *Z. Physik. Chem. 92*, 129–168.
Solc, K., & Stockmayer, W. H. (1973) *Int. J. Chem. Kinet. 5*, 733–752.
Stokes, R. H., & Weeks, I. A. (1964) *Aust. J. Chem. 17*, 304–309.
Straus, D., Raines, R., Kawashima, E., Knowles, J. R., & Gilbert, W. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2272–2276.
Trentham, D. R., McMurray, C. H., & Pogson, C. I. (1969) *Biochem. J. 114*, 19–24.
Webb, M. R., Standring, D. N., & Knowles, J. R. (1977) *Biochemistry 16*, 2738–2741.
Welch, G. R., Somogyi, B., Matko, J., & Papp, S. (1983) *J. Theor. Biol. 100*, 211–238.
Whittenburg, S. L. (1983) *J. Theor. Biol. 100*, 675–683.

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).

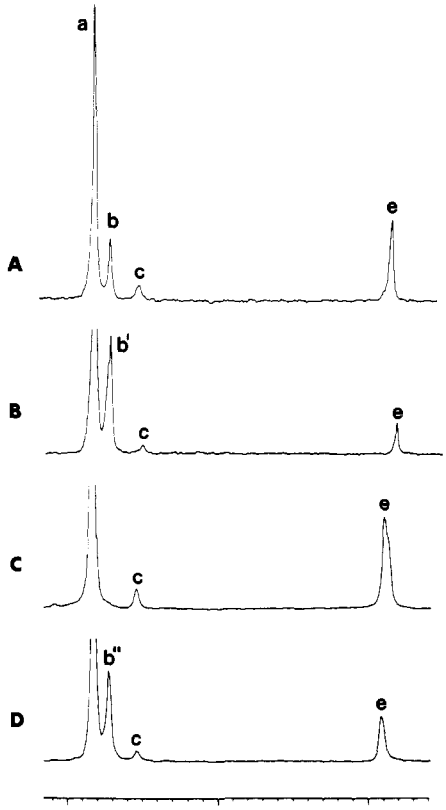


FIGURE 1: ^{31}P NMR spectra of glyceraldehyde phosphate (20–30 mM), pH 7.6, 30 °C. Peak assignments are given in Table I. Solvents used are (A) standard assay buffer [100 mM triethanolamine hydrochloride, pH 7.6, containing EDTA (5 mM)], (B) standard assay buffer plus 36% (w/w) glycerol, (C) water adjusted to pH 7.6, and (D) unbuffered aqueous 28% (w/w) glycerol solution at pH 7.6.

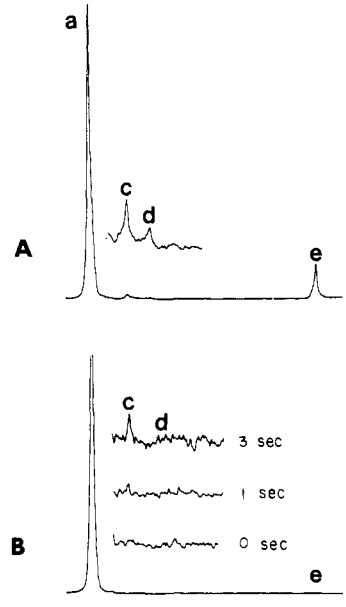


FIGURE 2: ^{31}P NMR spectra of glyceraldehyde phosphate (54 mM) in water, pH 7.6, at 5 °C. Peak assignments are given in Table I. (A) Equilibrium spectrum; (B) difference spectra at incremental delay times, t, after inversion of peak a. Insets are enlarged 8-fold.

NMR Spectral Conditions. All Fourier-transformed ^{31}P NMR spectra were proton decoupled and were acquired at 121 MHz on a Bruker WM-300 WB spectrometer. Samples were contained in 10-mm tubes with a coaxial 5-mm tube of D_2O as a field-frequency lock. Spectra were acquired at 30 °C except as noted. Temperature was measured with a thermocouple digital thermometer and regulated with a flow of dry nitrogen. A 90° pulse width and a 7-s delay between pulses were used. Gated proton decoupling was employed to minimize nuclear Overhauser enhancements. Chemical shifts were measured digitally with reference to 85% (w/w) phosphoric acid at 30 °C. Intensities were measured digitally, and integrals were determined by cutting and weighing. Up to 300 transients were accumulated, and line broadening of 0.6 Hz was applied to the free induction decay before Fourier transformation.

Inversion transfer spectra were acquired with a 180° DANTE pulse sequence (Morris & Freeman, 1978). Scans were alternated between inverted and noninverted conditions in order to obtain the unperturbed spectra concurrently. Difference spectra were obtained by subtracting the perturbed from the unperturbed free induction decay before Fourier transformation. A total of 128 transients were accumulated for each spectrum, and line broadening of 1.6 Hz was applied.

Table I: Glyceraldehyde Phosphate ^{31}P NMR Spectral Assignments

peak	δ (ppm)	assignment
a	4.8	hydrate
b	4.7	triethanolamine adduct
b′	4.7	peaks b and b″
b″	4.7	glycerol adduct
c	4.5	free aldehyde
d	4.2[a]	dimer
e	2.9	P_i

[a] At 5 °C. All other values are reported for 30 °C. All shifts are relative to 85% (w/w) phosphoric acid at 30 °C.

Table II: Equilibrium Constants for the Isomerase-Catalyzed Reaction in Glycerol Buffers of Different Viscosity[a]

% glycerol	η/η^0	K_{GAP}(measd)[b]	$1/K_{eq}$(calcd)[c]
0	1.0	0.047 ± 0.005	23
10	1.3	0.038 ± 0.004	19
18	1.6	0.035 ± 0.004	17
27	2.0	0.025 ± 0.003	12
34	2.6	0.017 ± 0.002	9

[a] The concentrations of additive are in percent (w/w). The values of the relative viscosity, η/η^0, were measured at 30 °C. K_{DHAP}(measd) = 2.1 at all glycerol concentrations listed. [b] Measured by NMR peak integration. [c] Calculated with eq 2.

RESULTS

Dihydroxyacetone Phosphate. The spectrum of dihydroxyacetone phosphate species showed no change upon the addition of glycerol. The ketone and hydrate were the only species found. On the basis of the peak assignments of Gray (1971), the value of K_{DHAP} was found to be 2.1 at 30 °C.

Glyceraldehyde Phosphate. Spectra of glyceraldehyde phosphate are shown in Figure 1. Spectrum 1A is taken in 0.1 M triethanolamine hydrochloride buffer, pH 7.6. Spectrum 1B is the same as spectrum 1A except for the addition of 36% (w/w) glycerol. The largest peak, a, is assigned to the hydrate, as glyceraldehyde phosphate has been previously shown to exist predominantly in this form (Trentham et al., 1969). Peak b (spectrum 1A) increased in area upon addition of glycerol (spectrum 1B: peak b′). In order to identify any nucleophilic adducts of glyceraldehyde phosphate, spectra were taken of glyceraldehyde phosphate in water (spectrum 1C) and in unbuffered 28% (w/w) glycerol (spectrum 1D). Peak b disappeared when triethanolamine was excluded from the solvent (spectrum 1C) but reappeared as peak b″ when glycerol alone was included (spectrum 1D). From these results we conclude that peak b′ (spectrum 1B) contains two resonances: peak b, due to the glyceraldehyde phosphate–triethanolamine

species, and peak b″, due to the glyceraldehyde phosphate–glycerol species. Peak e derives from inorganic phosphate that results from the slow decomposition of the triose phosphate sample.

In order to confirm that the remaining peak c is that from the free aldehyde, the existence of chemical exchange between the hydrate and the species resulting in peak c was investigated by inversion transfer. This experiment was done at 5 °C in order to minimize glyceraldehyde phosphate decomposition. Spectra taken at temperatures between 5 and 30 °C showed no change in the relative chemical shifts of peaks a and c. The spectrum of glyceraldehyde phosphate in water at 5 °C is shown in Figure 2A. At this temperature a new peak, d, appeared. The relative area of this peak decreases with decreasing concentration of total glyceraldehyde phosphate, identifying this peak as deriving from a glyceraldehyde phosphate dimer. Inversion transfer difference spectra are shown in Figure 2B, where peak a is irradiated. Magnetization was clearly transferred to peak c, which can therefore be assigned to the free aldehyde form of glyceraldehyde phosphate. All peak assignments are summarized in Table I.

Perturbation of K_{GAP} by Addition of Glycerol. On the basis of the above assignments, K_{GAP} was measured by integration of spectra of glyceraldehyde phosphate in buffers containing between 0 and 36% (w/w) glycerol. Over this range of glycerol concentrations, K_{GAP} decreases from 0.047 to 0.017. These results are given in Table II.

Discussion

Dihydroxyacetone phosphate exists in solution as a mixture of the keto form and the hydrate in a ratio of 2.1:1, and it forms no other species in significant amounts upon the addition of glycerol. In contrast, glyceraldehyde phosphate exists as a mixture of up to five different species. In addition to the free aldehyde and its hydrate, glyceraldehyde phosphate forms a complex with the nucleophilic buffer, triethanolamine. Upon addition of glycerol, glyceraldehyde phosphate forms a fourth species. Though these glyceraldehyde phosphate adducts have not been fully characterized, we can conclude that they are hemiacetals formed by the attack of hydroxyl groups of triethanolamine or glycerol on the aldehyde carbonyl center. Ketones are far less susceptible to nucleophilic attack, and it is not surprising that corresponding hemiketals of dihydroxyacetone phosphate are not generated. Finally, below 10 °C, a fifth glyceraldehyde phosphate species, a dimer, is formed. Since dimers are entropically disfavored relative to monomers, it is reasonable that the dimer only becomes detectable at low temperatures.

Under our standard kinetic assay conditions, glyceraldehyde phosphate exists as three species. When glycerol is added as a cosolvent, one additional species is formed. According to Le Chatelier's principle, the formation of this new species will decrease the overall free energy of glyceraldehyde phosphate relative to that of dihydroxyacetone phosphate, shifting K_{eq} in favor of glyceraldehyde phosphate (see Scheme I). Knowing the values of K_{DHAP}, K_{GAP}, and K_{TIM}, the value of K_{eq} can be calculated from eq 2. The values of K_{DHAP} and K_{GAP} have been measured by ^{31}P NMR as a function of glycerol concentration (Table II). We assume that the value of K_{TIM} is independent of glycerol concentration, since any change in K_{TIM} would require a glycerol-induced stabilization of either the glyceraldehyde phosphate free aldehyde or the dihydroxyacetone phosphate free ketone with respect to the other. Such a discriminatory effect on two such similar molecules seems unlikely. Glycerol might cause changes in the free energies of *both* "active" triose phosphates, but such changes would most likely be of about the same magnitude and thus not alter K_{TIM} significantly. The literature value for K_{TIM} of 0.003 (Knowles & Albery, 1977) and the above data yield the calculated values of $1/K_{eq}$ at different glycerol concentrations that are listed in Table II. These values should be compared with those obtained by direct measurement and by application of the Haldane relationship to the kinetic results that are listed in Table IV of the main paper. The agreement is gratifying.

The present results indicate that the addition of glycerol perturbs the triose phosphate equilibrium by forming a new glyceraldehyde phosphate species that selectively stabilizes total glyceraldehyde phosphate relative to total dihydroxyacetone phosphate. The dependence of K_{eq} on glycerol concentration therefore predicts that the measured kinetic parameters of triosephosphate isomerase will exhibit a dependence on glycerol concentration in addition to any manifestations of a viscosogenic effect. This dependence is used in the main paper to aid in the interpretation of the kinetics of the isomerase-catalyzed reaction observed in the presence of glycerol.

References

Alger, J. R., & Schulman, R. G. (1984) *Q. Rev. Biophys. 17*, 83–124.

Brown, T. R., & Ogawa, S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3627–3631.

Gray, G. R. (1971) *Biochemistry 10*, 4705–4711.

Knowles, J. R., & Albery, W. J. (1977) *Acc. Chem. Res. 10*, 105–111.

Morris, G. A., & Freeman, R. (1978) *J. Magn. Reson. 29*, 433–462.

Reynolds, S. J., Yates, D. W., & Pogson, C. I. (1971) *Biochem. J. 122*, 285–297.

Trentham, D. R., Murray, C. H., & Pogson, C. I. (1969) *Biochem. J. 114*, 19–24.

Webb, M. R., Standring, D. N., & Knowles, J. R. (1977) *Biochemistry 16*, 2738–2741.