Segmental Motion in Catalysis: Investigation of a Hydrogen Bond Critical for Loop Closure in the Reaction of Triosephosphate Isomerase†

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ABSTRACT: A residue essential for proper closure of the active-site loop in the reaction catalyzed by triosephosphate isomerase is tyrosine-208, the hydroxyl group of which forms a hydrogen bond with the amide nitrogen of alanine-176, a component of the loop. Both residues are conserved, and mutagenesis of the tyrosine to phenylalanine results in a 2000-fold drop in the catalytic activity (k_{cat}/K_m) of the enzyme compared to the wild-type isomerase. The nature of the closure process has been elucidated from both viscosity dependence and primary isotope effects. The reaction catalyzed by the mutant enzyme shows a viscosity dependence using glycerol as the viscosogen. This dependence can be attributed to the rate-limiting motion of the active-site loop between the "open" and the "closed" conformations. Furthermore, a large primary isotope effect is observed with [1-^{2}H]dihydroxyacetone phosphate as substrate [$(k_{cat}/K_m)^H/(k_{cat}/K_m)^D = 6 \pm 1$]. The range of isotopic experiments that were earlier used to delineate the energetics of the wild-type isomerase has provided the free energy profile of the mutant enzyme. Comparison of the energetics of the wild-type and mutant enzymes shows that only the transition states flanking the enediol intermediate have been substantially affected. The results suggest either that loop closure and deprotonation are coupled and occur in the *same* rate-limiting step or that these two processes happen sequentially but interdependently. This finding is consistent with structural information that indicates that the catalytic base glutamate-165 moves 2 Å toward the substrate upon loop closure. The motion of the mobile loop of triosephosphate isomerase is therefore linked to substrate deprotonation by the catalytic base glutamate-165.

In the preceding paper (Sampson & Knowles, 1992), we examined the importance of several hydrogen bonds in the stabilization of the two conformations of the active-site loop of triosephosphate isomerase (TIM).[1] It was concluded that the most important catalytic aspect of the mobile loop lies in the stabilization of the "closed" form. To elucidate the nature of the closing process, the mechanism of one mutant enzyme, constructed by substitution of phenylalanine for tyrosine at position 208, has been examined in detail.

In the "closed" form of the wild-type enzyme, the tyrosine hydroxyl oxygen of residue 208 is 2.9 Å from the amide nitrogen of alanine-176, a loop residue (Lolis & Petsko, 1990). This distance and the appropriate placing of the two heteroatoms suggest that a hydrogen bond exists between them in the "closed" conformation. In contrast, the tyrosine hydroxyl oxygen is 5.0 Å away from the alanine-176 amide nitrogen in the "open" structure (Lolis et al., 1990). In the mutant Y208F protein, where this hydrogen bond interaction is impossible, a large reduction in catalytic activity is seen. This loss is surprising since no direct interactions with the substrate have been disturbed, and only one of four hydrogen bonds that form upon closure of the loop has been removed. We have determined if the lowered catalytic potency of the Y208F enzyme is the result either of a looser "grip" on the substrate/intermediate in the closed form or of a slowed loop movement. The first possibility would be reminiscent of the behavior of the loop-deletion mutant constructed by Pompliano et al. (1990) which catalyzed the formation of substantial amounts of methylglyoxal, a decomposition product formed from the enediol intermediate by elimination of inorganic phosphate. The second possibility of slow loop movement implies that the hydrogen bond between the tyrosine hydroxyl and the amide NH forms in the transition state of loop closure. Removal of this hydrogen bond would be expected to raise the energy barrier for the formation of the "closed" species, and to impair catalysis without necessarily involving the loss of the intermediate from the active site. We report here the kinetic characterization of the mutant Y208F triosephosphate isomerase, and discuss the implications for the mechanism of loop movement during catalysis by this enzyme.

EXPERIMENTAL PROCEDURES

Materials. (*RS*)-Glyceraldehyde 3-phosphate (diethylacetal, monobarium salt), dihydroxyacetone phosphate (lithium salt), NADH, and triosephosphate isomerase (yeast) were from Sigma Chemical Co. (St. Louis, MO). The viscosogenic agents were ficoll (M_r 4 × 10^5) from Pharmacia Fine Chemicals (Piscataway, NJ), glycerol from Fisher Chemical (Fairlawn, NJ), and poly(ethylene glycol) (M_r 8 × 10^3) from United States Biochemical (Cleveland, OH). Aldolase (rabbit muscle), phosphoglycerate kinase (yeast), pyruvate kinase (rabbit muscle), glyoxylase I (yeast), alkaline phosphatase (calf intestine), glycerophosphate dehydrogenase (rabbit muscle), and glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle) were obtained as ammonium sulfate suspensions from Boehringer Mannheim Biochemicals (Indianapolis, IN). The H95Q isomerase (yeast) was prepared by Dr. E. Komives as described by Komives et al. (1991). Traces of contaminating isomerase activity were removed from the dehydrogenases and kinases by prior treatment with bromohydroxyacetone phosphate as described by Plaut and Knowles (1972). Bromohydroxyacetone phosphate was prepared by

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[1] Abbreviations: TIM, triosephosphate isomerase; PGH, phosphoglycolohydroxamate; GAP, (*R*)-glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate.

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Dr. E. Komives according to the method of de la Mare et al. (1972). Phosphoglycolohydroxamate [bis(cyclohexylammonium) salt] was synthesized by Dr. J. G. Belasco as described in Belasco and Knowles (1980). [1(*R*)-^{3}H]Dihydroxyacetone phosphate (9 Ci/mol) was prepared by Dr. R. T. Raines according to the method of Herlihy et al. (1976). Phospho-[1-^{14}C]glycerate (14.1 Ci/mol) was prepared by Dr. D. L. Pompliano according to the method of Guilford et al. (1987). [1-^{14}C]Glyceraldehyde 3-phosphate (0.04 Ci/mol) and [1-^{14}C]-dihydroxyacetone phosphate (49 Ci/mol) were prepared by Dr. D. L. Pompliano according to the method of Nickbarg et al. (1988). [2-^{3}H]Glyceraldehyde 3-phosphate was prepared according to the method of Nickbarg et al. (1988). Tritiated water (0.9 Ci/mL) was from Amersham (Chicago, IL). Flo Scint IV scintillation cocktail was obtained from Radiomatic Instruments & Chemical Co. (Tampa, FL). All other chemicals and reagents were of a commercial reagent grade or better and were used without further purification. The mutant Y208F yeast isomerase was constructed, expressed, and purified as described in Sampson and Knowles (1992).

Methods. Samples for radiochemical analysis (5–100 μL) were dissolved in scintillation cocktail (5–8 mL) and counted in a Beckman LS1801 automatic liquid scintillation counter. Scintillation counting for both ^{3}H and ^{14}C was done after calibration with Beckman counting standards and by use of the double-label counting programs supplied with the instrument. pH was measured with a Corning 245 pH meter fitted with a Sigma E5634 electrode and calibrated at room temperature. Ultraviolet and visible absorbances were measured on a thermostated Varian CARY3 spectrophotometer.

Mixtures of radiolabeled 3-phospho-D-glycerate and *sn*-glycerol 3-phosphate were separated on a Pharmacia Mono-Q HR 5/5 column. The separation involved an isocratic wash (4 min) with 10 mM triethylammonium formate, pH 3.8, followed by a linear gradient (10–1000 mM over 20 min) of triethylammonium formate buffer, pH 3.8, at 2 mL/min. Column eluent was mixed on-line with scintillation fluid and monitored with a flow-through scintillation counter (Flo-One/Beta, Model CT from Radiomatic Instruments and Chemical Co.). Fractions were collected and counted in the Beckman scintillation counter.

The concentration of isomerase was based upon Bradford assay using wild-type yeast isomerase as a standard. A subunit molecular weight of 26 500 was assumed (Putnam et al., 1972), and kinetic parameters are quoted for a single subunit. The kinetic parameters were calculated with a nonlinear least-squares fitting program, Grafit (Elsevier Publishing). All enzyme-catalyzed reactions were conducted at 30 °C.

Inorganic phosphate content was determined by the method of Ames (1966). Methylglyoxal was quantitated by end-point assay using glyoxylase I, as described by Racker (1957).

Preparation of Viscosogenic Buffers and Measurement of Viscosity. Each viscosogenic buffer was 0.1 M triethanolamine hydrochloride, pH 7.6, containing EDTA (10 mM), dithiothreitol (1 mM), and the viscosogenic agent. The viscosity (η) of each assay mixture was determined at 30 °C as the product of the kinematic viscosity, η/ρ, as measured with an Ostwald viscometer, and the density, ρ. Viscosities are reported relative to 0.1 M triethanolamine hydrochloride buffer, pH 7.6, containing EDTA (10 mM) and dithiothreitol (1 mM).

[1(R)-^{2}H]Dihydroxyacetone Phosphate. [1(*R*)-^{2}H]Dihydroxyacetone phosphate was prepared by equilibration of dihydroxyacetone with ^2H_2O and yeast triosephosphate isomerase essentially as described by Leadlay et al. (1976), except that proteins were removed at each step by ultrafiltration rather than by chromatography. The final product was purified by anion exchange as previously described.

Table I: Steady-State Kinetic Parameters for Wild-Type and Mutant Isomerases

parameter[a]	wild type[b]	mutant Y208P[b]	units	wild type/mutant Y208F
k_{cat^-}	(8 ± 0.4) × 10^3	7.5 ± 0.1	s^{-1}	1100
K_{m^-}	1.3 ± 0.1	2.9 ± 0.1	mM	0.45
K_{m^-}(unhyd)[c]	0.048 ± 0.004	0.11 ± 0.004	mM	0.45
k_{cat^+}	(7.4 ± 0.2) × 10^2	1.2 ± 0.2	s^{-1}	620
K_{m^+}	2.2 ± 0.2	7.9 ± 0.9	mM	0.28
K_{m^+}(unhyd)[c]	1.3 ± 0.1	4.8 ± 0.5	mM	0.28
K_i(PGH)	16 ± 2	2800 ± 400	μM	0.0057
K_i($HAsO_4^{2-}$)	9.6 ± 0.3	25	mM	0.38
K_{eq}[d]	18 ± 2	17 ± 3		1

[a] A(–) superscript indicates (*R*)-glyceraldehyde 3-phosphate as the substrate. A (+) superscript indicates dihydroxyacetone phosphate as the substrate. [b] All experiments were conducted at 30 °C in 100 mM triethanolamine hydrochloride buffer, pH 7.6, containing EDTA (10 mM). [c] Values for the unhydrated form calculated as in Albery and Knowles (1976b). [d] The overall equilibrium constant determined from the Haldane relationship.

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

η/η^0	wild-type isomerase	Y208F isomerase	H95Q isomerase
1.0	1.0	1.0	1.0
1.4	1.7 ± 0.2	1.1 ± 0.1	
1.6			1.3 ± 0.1
1.7	1.7 ± 0.1	1.6 ± 0.2	
2.1	2.3 ± 0.1	1.84 ± 0.05	1.2 ± 0.06
2.8	3.0 ± 0.1	2.2 ± 0.2	
3.1	3.4 ± 0.3		
3.2	3.7 ± 0.4	2.8 ± 0.3	1.5 ± 0.1

[a] (*R*)-Glyceraldehyde 3-phosphate is the substrate. Each k_{cat}/K_m value was determined from runs at six different substrate concentrations at 30 °C, pH 7.6.

RESULTS

The steady-state kinetic parameters for the wild-type and mutant Y208F enzymes are listed in Table I. To check the stability of the triose phosphates in the presence of Y208F isomerase, the mutant isomerase was incubated with substrate under reversible conditions (that is, in the absence of coupling enzymes or their cofactors). The observed loss of triose phosphates did not differ significantly from control incubations either containing no enzyme or containing an equal number of units of wild-type enzyme. This finding was confirmed by the absence of assayable methylglyoxal or inorganic phosphate at the end of these incubations.

The values of k_{cat^-}/K_{m^-} were determined in the presence of varying levels of glycerol for the reaction of (*R*)-glyceraldehyde 3-phosphate catalyzed by the wild-type isomerase, by the mutant Y208F enzyme, and by the mutant H95Q enzyme. Each value of k_{cat^-}/K_{m^-} is reported in Table II relative to $(k_{cat^-}/K_{m^-})^0$ determined concurrently. [The "minus" superscript refers to (*R*)-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 values of k_{cat^+}/K_{m^+} were determined in the presence of varying levels of glycerol for the reaction of dihydroxyacetone phosphate catalyzed by the wild-type isomerase, by the mutant Y208F enzyme, and by the mutant H95Q enzyme. Each value of k_{cat^+}/K_{m^+} is reported in Table III relative to $(k_{cat^+}/K_{m^+})^0$ determined concurrently. (The "plus" superscript refers to dihydroxyacetone phosphate as substrate.)

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

η/η^0	wild-type isomerase	Y208F isomerase	H95Q isomerase
1.0	1.0	1.0	1.0
1.6	1.1 ± 0.1		0.77 ± 0.03
2.1	1.3 ± 0.2	1.0 ± 0.03	0.59 ± 0.03
2.8	1.8 ± 0.6	1.0 ± 0.1	0.56 ± 0.06
3.2	2.2 ± 0.2	0.9 ± 0.2	0.53 ± 0.08

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

Table IV: Values of k_{cat}^0/k_{cat} for the Mutant H95Q Isomerase with Glycerol as the Viscosogenic Agent

η/η^0	$k_{cat^-}^0/k_{cat^-}$[a]	$k_{cat^+}^0/k_{cat^+}$[b]
1.0	1.0	1.0
1.6	1.2 ± 0.05	0.78 ± 0.01
2.1	1.1 ± 0.03	1.0 ● 0.02
2.8		1.0 ± 0.02
3.2	1.2 ± 0.04	0.95 ● 0.01

[a] (*R*)-Glyceraldehyde 3-phosphate is the substrate. The absolute value for $k_{cat^-}^0$ was found to be 4.8 ± 0.1 s^{-1}.

Table V: Variation in the Specific Radioactivity of Substrate [1(*R*)-^{3}H, 1-^{14}C]Dihydroxyacetone Phosphate (*s*) or of Substrate [2-^{3}H, 1-^{14}C]Glyceraldehyde 3-Phosphate (*p*) in Reactions Catalyzed by the Mutant Y208F Yeast Isomerase

extent of reaction of s $(1-r)$	^{3}H content in remaining substrate (s/s_0)
0	1.0
0.44	1.4
0.91	8.7

extent of reaction of p $(1-r)$	^{3}H content in remaining substrate (p/p_0)
0	1.0
0.30	1.3
0.50	1.4
0.76	2.2

For both wild-type and mutant isomerases, the values of $(k_{cat}/K_m)^0/(k_{cat}/K_m)$ showed no dependence on η/η^0 for *polymeric* viscosogenic additives [e.g., ficoll or poly(ethylene glycol)] at relative viscosities up to 4. The values of k_{cat^-} and k_{cat^+} in the presence of various concentrations of glycerol were also determined for the mutant H95Q enzyme and are listed in Table IV.

The kinetic effects of deuterium substitution were investigated with [1(*R*)-^{2}H]dihydroxyacetone phosphate. Comparison of the results generated from the least-squares fitting program ISOVKVC (Cleland, 1979) shows that the primary isotope effects are identical for V and V/K. The deuterium isotope effect on k_{cat}^H/k_{cat}^D is 6 ± 1.

The extent of tritium transfer from the substrate [1(*R*)-^{3}H, 1-^{14}C]dihydroxyacetone phosphate to the ultimate product 3-phosphoglycerate catalyzed by the mutant Y208F isomerase was less than 1% after the reaction was 90% complete. The results for the relative specific radioactivity of the remaining substrate (s/s_0) at two extents of reaction are listed in Table V. The extent of tritium transfer from the substrate [2-^{3}H, 1-^{14}C]glyceraldehyde 3-phosphate in the reverse reaction catalyzed by the mutant isomerase was less than 4%. The relative specific radioactivity of the remaining substrate (p/p_0) at various extents of reaction is listed in Table V.

With [1-^{14}C]dihydroxyacetone phosphate as substrate, two exchange-conversion experiments were performed for the mutant Y208F isomerase in tritiated water at various extents of reaction. The values obtained for the relative specific radioactivity of the remaining dihydroxyacetone phosphate, *s* (expressed as a fraction of the specific radioactivity of the solvent *x*), as a function of the extent of reaction $(1-r)$ are listed in Table VI. The specific radioactivity of tritium in product glyceraldehyde 3-phosphate (which was converted in situ to 3-phosphoglycerate), *p* (expressed as a fraction of the specific radioactivity of the solvent *x*), was determined and is shown at various extents of reaction in Table VI. With [1-^{14}C]glyceraldehyde 3-phosphate as substrate, two exchange-conversion experiments were performed for the mutant isomerase in tritiated water at various extents of reaction. The values obtained for the relative specific radioactivity of the remaining glyceraldehyde 3-phosphate (which was converted to 3-phosphoglycerate before analysis), *p* (expressed as a fraction of *x*), are listed in Table VII. The specific radioactivity of product dihydroxyacetone phosphate (converted in situ to glycerol phosphate), *s* (expressed as a fraction of *x*), is listed in Table VII for various extents of reaction.

Table VI: Mutant Y208F Isomerase Catalyzed Incorporation of Solvent Tritium into Remaining Substrate Dihydroxyacetone Phosphate and into Product Phosphoglycerate, during the Mutant Y208F Isomerase Catalyzed Reaction of Dihydroxyacetone Phosphate

fractional extent of reaction $(1-r)$	^{3}H content in remaining substrate (s/x)	^{3}H content in product (p/x)
0.0	0.0	
0.25	0.021	0.10
0.50	ND[a]	0.12
0.52	0.026	0.11
0.56	0.035	0.16
0.76	0.096	0.12
0.79	0.088	0.12
1.0		0.12

[a] ND, not determined.

Table VII: Mutant Y208F Isomerase Catalyzed Incorporation of Solvent Tritium into Remaining Substrate Glyceraldehyde 3-Phosphate and into Product Glycerol Phosphate, during the Mutant Y208F Isomerase Catalyzed Reaction of Glyceraldehyde 3-Phosphate

fractional extent of reaction $(1-r)$	^{3}H content in remaining substrate (p/x)	^{3}H content in product (s/x)
0.0	0.0	
0.45	0.056	0.037
0.50	0.073	ND[a]
0.59	0.119	0.063
0.68	0.125	0.052
0.78	0.218	0.041
0.98	0.688	0.046
1.0		0.050

[a] ND, not determined.

DISCUSSION

Triosephosphate isomerase catalyzes the simple isomerization of an α-hydroxy ketone to an α-hydroxy aldehyde, and has evolved as a catalyst to the limit of diffusion control. The deprotonation and protonation steps occur relatively rapidly, and diffusion of the thermodynamically less stable substrate glyceraldehyde 3-phosphate, on and off the enzyme, limits the overall rate of catalysis. A particularly interesting feature of the catalytic mechanism is the existence of a loop of about 10 amino acids that clamps down upon substrate bound at the active site.

The current picture of the function of this loop is as a structured entity (Joseph et al., 1990) that sequesters substrate from the solvent and selectively stabilizes the reactive

intermediate in the isomerization reaction. Hydrogen bonds between the backbone amides of the loop and the substrates' peripheral phosphate oxygens appear to stabilize the liganded enzyme. The essential catalytic function of the loop has been illuminated by the specific deletion of four residues (Pompliano et al., 1990), a truncation that led not only to a precipitous drop in catalytic activity but also to a large reduction in "throughput" efficiency. Thus, the mutant loop-truncated enzyme loses the intermediate enediol to the solvent 5 times more frequently than carbon protonation occurs to form the product. By comparison, the wild-type enzyme loses the intermediate less than once in every 10^5 turnovers (Richard, 1991).

Subsequent mutagenesis experiments (Sampson & Knowles, 1992) have defined the catalytic importance of the stability of the "closed" form of the enzyme. Kinetic characterization of the mutant Y208F enzyme then allowed a clearer picture of the loop closure to be drawn. The mutant Y208F enzyme is reduced more than 1000-fold in catalytic activity, and binds the intermediate analogue PGH 200-fold less tightly than does the wild-type enzyme. On first inspection, these data lead one to believe that removal of a single hydrogen bond between the mobile loop and the bulk of the protein has a deleterious effect on catalysis because the mutant enzyme cannot now keep hold of the reaction intermediate. This hypothesis was readily tested by determining the rate of triose phosphate decomposition in the presence of mutant enzyme. Unexpectedly, none of the substrate decomposition product, methylglyoxal, was detected.

If the mutant protein has not loosened its grasp on the intermediate, what other effects might this removal of a single tyrosine hydroxyl wreak on catalysis? We can imagine that the rate of complexation of substrate could be impaired by introducing a mutation that interferes with loop closure. Alternatively, the enolization process itself could be slowed due to a decreased ability to stabilize the reaction intermediate. Perhaps the "closed" form is somehow distorted relative to the wild-type enzyme and the reaction intermediate is less well stabilized. Finally, since a single mutation may have multiple effects, some combination of these possibilities could explain the observed kinetic effect.

The mobile loop of TIM moves a relatively large distance through solution upon substrate binding (about 7 Å for the central residue, T172), and we reasoned that this motion might be sensitive to variation in the solvent viscosity. Furthermore, if the transition state for loop closure is partially rate-limiting, then the overall rate of catalysis should also be affected. By examination of the effect of solvent viscosity on the catalytic rate, therefore, the result of removing a single tyrosine hydroxyl may be ascertained. The viscosity dependence of wild-type TIM has been previously studied with the chicken enzyme (Blacklow et al., 1988). In that case, it was known from independent experiments that the rate-limiting transition state for the overall reaction involves product release. Demonstration that the viscosity dependence of the reaction conformed to the prediction of the Stokes–Einstein equation for a cleanly diffusion-limited reaction verified that product release is indeed rate-limiting and that this process is diffusion-controlled. It is reasonable to assume that any single mutation in the enzyme will not affect the rate of diffusion of the product or substrate to the active site. The rate-limiting transition state for the Y208F enzyme (which is more than 10^3 times slower than the wild type) will, therefore, not involve the "on" or "off" steps for substrate or for product. If, then, we were to observe a viscosity dependence of the mutant Y208F enzyme, we should have to attribute it to some solvent-dependent motion of a

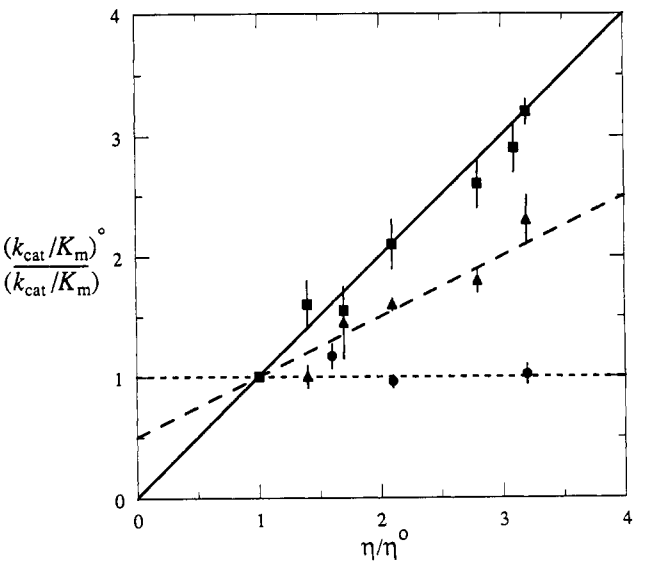

FIGURE 1: Plot of the normalized values for the reciprocal of the relative second-order rate constant for wild-type (■), mutant Y208F (▲), and mutant H95Q (●) isomerases with glyceraldehyde 3-phosphate as substrate and glycerol as viscosogenic agent. The slope (0.22) of the least-squares regression line for the variation of $(k_{cat}/K_m)^0/(k_{cat}/K_m)$ with η/η^0 for the mutant H95Q isomerase was used to normalize each value of $(k_{cat}/K_m)^0/(k_{cat}/K_m)$ with the expression $[(k_{cat}/K_m)^0/(k_{cat}/K_m)]_{norm} = [(k_{cat}/K_m)^0/(k_{cat}/K_m)]_{obsd} - 0.22(\eta/\eta^0 - 1)$. The primary data were taken from Table II. The slope of the regression line for the normalized results with the mutant H95Q isomerase is 0.00 ± 0.04, with the mutant Y208F isomerase is 0.51 ± 0.05, and with the wild-type isomerase is 0.96 ± 0.03.

liganded enzyme *complex*, the most reasonable of which would involve movement of the flexible loop.

As was observed in an earlier study (Blacklow et al., 1988), the viscosogen glycerol alters the equilibrium between the hydrated and unhydrated forms of the substrates, largely by forming hemiacetals with GAP. It is necessary to correct for these effects on the rate. Since it is known that the chemical steps in catalysis by the very slow mutant H95Q enzyme are cleanly rate-limiting (Nickbarg et al., 1988), the behavior of this enzyme can be used as a control for all the nonviscosity solvent effects that are introduced by the addition of the viscosogen glycerol. Indeed, the independence of k_{cat} from the presence of glycerol confirms that the chemical steps of H95Q enzyme catalysis are not affected by viscosity and that this mutant enzyme does serve as a proper control. [A complete discussion of these peripheral effects, and independent measurements of the perturbation in substrate equilibria, is presented in Blacklow et al. (1988).] The normalized results both for the wild-type yeast enzyme and for the Y208F mutant isomerase are presented in Figures 1 and 2. As expected from our earlier experiments and from the known free energy profile of the wild-type yeast isomerase (Nickbarg & Knowles, 1988), the wild-type enzyme reaction is diffusion-limited. The data for the catalyzed reaction in both directions agree well with the prediction of the Stokes–Einstein equation for the bimolecular reaction of two freely diffusing species. The gradients of plots of the relative second-order rate constant versus the relative solvent viscosity are 1.0 ± 0.1 and 0.96 ± 0.03 for DHAP and GAP as substrate, respectively. Interestingly, the mutant Y208F enzyme *also shows a viscosity dependence*, with a gradient of about 0.5 in each direction. It is clear that solvent viscosity modulates the catalytic activity of the Y208F enzyme.

Although the gradients of 0.5 in Figures 1 and 2 might suggest that the reaction of the Y208F enzyme is only 50% diffusion-controlled, loop closure is an *intra*molecular diffusive

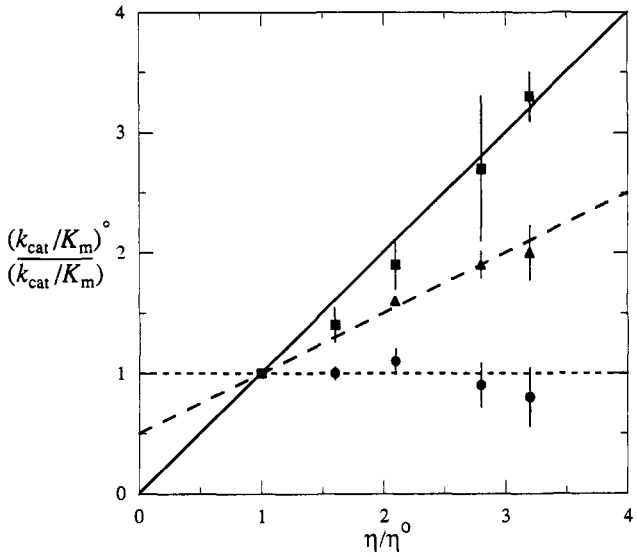

FIGURE 2: Plot of the normalized values for the reciprocal of the relative second-order rate constant for wild-type (■), mutant Y208F (▲), and mutant H95Q (●) isomerases with dihydroxyacetone phosphate as substrate and glycerol as viscosogenic agent. The slope (0.52) of the least-squares regression line for the variation of $(k_{cat}/K_m)/(k_{cat}/K_m)^0$ with η/η^0 for the mutant H95Q isomerase was used to normalize each value of $(k_{cat}/K_m)^0/(k_{cat}/K_m)$. For the H95Q mutant isomerase, the expression $[(k_{cat}/K_m)/(k_{cat}/K_m)^0]_{norm} = [(k_{cat}/K_m)/(k_{cat}/K_m)^0]_{obsd} - 0.52(\eta/\eta^0 - 1)$ was used to derive the normalized values of $(k_{cat}/K_m)^0/(k_{cat}/K_m)$. For wild-type and mutant Y208F isomerases, the expression $[(k_{cat}/K_m)^0/(k_{cat}/K_m)]_{norm} = [(k_{cat}/K_m)^0/(k_{cat}/K_m)]_{obsd} + 0.52(\eta/\eta^0 - 1)$ was used. The primary data were taken from Table III. The slope of the regression line for the normalized results with the mutant H95Q isomerase is 0.00 ± 0.04, with the mutant Y208F isomerase is 0.53 ± 0.02, and with the wild-type isomerase is 1.0 ± 0.1.

process rather than an *inter*molecular process, and the Stokes–Einstein treatment must be modified to accommodate the motion of one part of a molecule with respect to another. Thus, although Einstein and Sutherland (1905) have demonstrated that the rate of molecular diffusion is inversely proportional to friction, the barrier to loop movement in the intramolecular case will derive from both the external and the internal friction, that is, the solution viscosity and the intrinsic energy barrier to bond rotation, respectively. The gradients of the plots of relative second-order rate constant versus relative viscosity simply reflect, therefore, the relative contributions of external and internal friction. There is no doubt, however, that solution viscosity has a palpable effect on the rate of the reaction catalyzed by the Y208F mutant enzyme.

Having shown that intramolecular protein movement has been slowed by removal of the tyrosine-208 hydroxyl group in the Y208F mutant, we asked if loop closure occurs simultaneously with substrate binding, if it occurs after substrate binding but before enolization, or if it occurs concomitantly with deprotonation of the substrate. Measurement of the primary isotope effect distinguishes among these possibilities. With DHAP as substrate, a large isotope effect, of 6 ± 1, was detected in both k_{cat}/K_m and k_{cat}. This finding eliminates the possibility that the overall rate-limiting barrier to the catalytic reaction arises before deprotonation of the substrate. *It appears that the viscosity-dependent process (which we have presumed is loop closure) and deprotonation of substrate occur together.*

The energetics of the reaction catalyzed by the mutant Y208F enzyme were probed by tracing the fate of radiolabel using tritiated substrates or tritiated water, in exchange-conversion and discrimination experiments. These experiments have been discussed earlier, both quantitatively (Albery & Knowles, 1976a) and qualitatively (Raines et al., 1986; Nickbarg & Knowles, 1988). The use of tritiated substrates confirmed that the enolizable proton of the substrate is exchanged rapidly with solvent during the course of the catalyzed reaction. Furthermore, the discrimination against substrate tritium results in an increase in the specific radioactivity of the remaining substrate. At the beginning of the reaction, the specific radioactivity reflects the primary kinetic isotope effect, and later in the reaction, the specific radioactivity reflects the partitioning of the intermediate between conversion to product and collapse back to substrate following the loss of tritium to the solvent. The enrichment of tritium in remaining substrate DHAP is substantial (9-fold) and indicates that the primary isotope effect dominates over washout. These experiments also demonstrate that the enolizable protons readily exchange with solvent water via the enzyme-bound enediol intermediate and allow interpretation of experiments in which solvent tritium appears in the substrate and the product. In a separate experiment, the relative barrier heights were determined by measuring the fraction of solvent tritium that appears in remaining substrate as a function of total product produced (exchange versus conversion) and the fraction of solvent tritium that appears in the product (discrimination). The primary tritium isotope effects determined from the discrimination experiments may be compared with the primary deuterium isotope effects using the Swain–Schaad relationship (Swain et al., 1958). For DHAP as substrate, the primary deuterium isotope effect calculated from the tritium discrimination experiments is 8 ± 1, which is in reasonable agreement with the directly measured value of 6 ± 1. For GAP as substrate, the calculated primary deuterium isotope effect is 4.5 ± 0.5. The exchange-conversion data demonstrate that the barriers flanking the intermediate dominate the overall rate of the catalyzed reaction. The complete free energy profile in Figure 3 was constructed as described in Raines et al. (1986) (Table VIII).

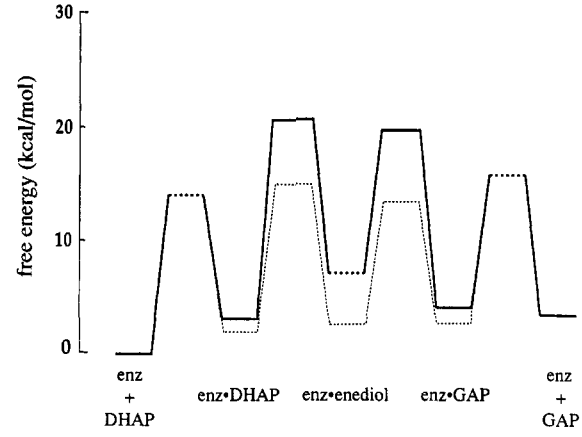

FIGURE 3: Free energy profiles for mutant Y208F isomerase (solid line) and wild-type yeast isomerase (dashed line). The profile for the wild-type enzyme is from Nickbarg and Knowles (1988). The profile for the mutant enzyme is derived from the raw data in Tables V–VII according to Raines et al. (1986). The relative free energy of bound enediol phosphate was estimated relative to that for the wild-type enzyme from the binding constants of phosphoglycolohydroxamate. The heavy dashed lines indicate free energy levels that are only estimates. The calculated rate constants are listed in Table VIII. The standard state used is 40 μM triose phosphates.

As can be seen in Figure 3, the transition states for enolization are cleanly rate-limiting for the mutant Y208F enzyme. The viscosity dependence of k_{cat}/K_m and of k_{cat} indicates that deprotonation of the substrate occurs in a solvent-dependent step, which, if this solvent-dependent step is loop closure, suggests that the α-proton is abstracted from substrate *as the loop closes*. By removal of the tyrosine hydroxyl group in the

Table VIII: Data for Free Energy Profile of Mutant Y208F Triosephosphate Isomerase[a]

value	ΔG (kcal/mol)
k_1[b] = 10^7 M^{-1} s^{-1}	14.1[c]
k_{-1} = 4.7×10^4 s^{-1}	11.3
k_2 = 1.4 s^{-1}	17.6
k_{-2} = 9.3×10^2 s^{-1}	13.6
k_3 = 4.4×10^3 s^{-1}	12.7
k_{-3} = 44 s^{-1}	15.5
k_4 = 2.2×10^4 s^{-1}	11.7
k_{-4}[b] = 2×10^8 M^{-1} s^{-1}	12.3[c]
$k_{-1}k_{-2}k_{-3}k_{-4}/k_1k_2k_3k_4$ = 285	3.4

[a] Determined at 30 °C. [b] From Albery and Knowles (1976b); values relate to the unhydrated substrate forms. [c] Calculated for a standard state of 40 μM.

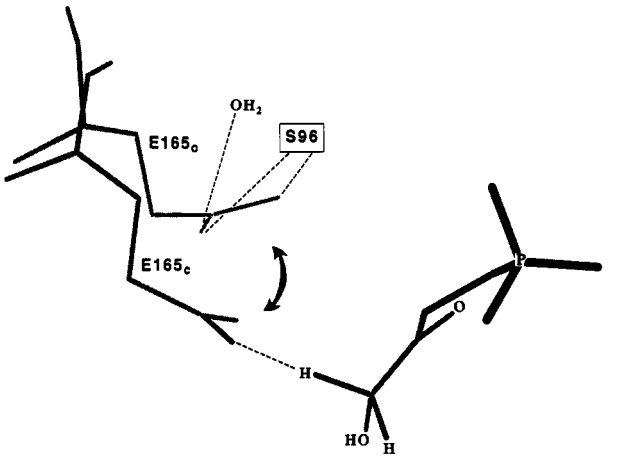

FIGURE 4: Glutamate-165 and a model of dihydroxyacetone phosphate in the active site of triosephosphate isomerase. The subscript c refers to the position of glutamate-165 in the "closed" form of the active-site loop; the subscript o refers to the position of glutamate-165 in the "open" form of the loop. In the "open" loop structure, the carboxylate of glutamate-165 is hydrogen-bonded to the hydroxyl and amide NH groups of serine-96, as well as to a water molecule. In the "closed" form, glutamate-165 is perfectly positioned to abstract the *pro-R* hydrogen of dihydroxyacetone phosphate, and is approximately 2 Å away from its position in the "open" form.

Y208F mutant, this process is slowed considerably, showing that formation of a hydrogen bond between the alanine-176 amide and the tyrosine-208 hydroxyl contributes to the driving force for loop closure. According to this view, the closure of the loop (which involves the movement of residues 166–176) would be coupled to a (smaller) movement of the catalytic base, glutamate-165. A viscosity increase would slow the movement of the loop, and affect the precise repositioning of the catalytic base for proton abstraction.

Upon examination of the "open" and "closed" structures of wild-type isomerase, the logic of coupling deprotonation and loop closure becomes quite attractive. Glutamate-165 lies at the edge of a β-sheet, two residues beyond the "end" of the mobile loop. This location allows glutamate-165 some flexibility in its positioning. Indeed, the carboxylate side chain of glutamate-165 is 2 Å closer to the C-1 and C-2 hydrogens of the substrate in the "closed" structure than in the "open" form (see Figure 4). One can see that as the loop closes, the hydrogen-bonding pattern of the carboxylate reorganizes, the glutamate moves toward the C-1 atom of the ligand, and enolization becomes possible. New hydrogen bonds form between the loop and the protein that stabilize the intermediate structure, and reprotonation at C-2 then occurs concomitantly with loop opening. An alternative interpretation that avoids any physical coupling of the motion of the loop and of glutamate-165 is that loop closure produces an inactive complex. This complex subsequently undergoes a relatively minor structural reorganization that allows enolization to occur. Such a reorganization would, of course, need to involve a "diffusional component" that is detected by the added viscosogen. While it is not possible to estimate the extent of protein movement in the solvent-dependent step on the basis of viscosity experiments alone, we are grateful to a reviewer for raising this possibility.

In summary, we have demonstrated that solvent-dependent motion of the protein *and* enolization are rate-limiting in the reaction catalyzed by the mutant Y208F isomerase. We suppose that some movement of the catalytic loop is involved in the solvent-dependent step. This motion is linked to the enolization of the substrate and serves to position the catalytic base glutamate-165, as well as to sequester the reaction intermediate. The movements of the catalytic loop and the catalytic base are tightly coupled, and exemplify the exact quasi-mechanical linking that is evidently necessary for optimal catalysis by this enzyme.

ADDED IN PROOF

To determine if the observation of both a viscosity dependence and a kinetic isotope effect in the reaction catalyzed by the mutant Y208F isomerase derives from the existence of two transition states (relating to protein movement and enolization) that are approximately equal in free energy, we have measured the solvent viscosity dependence of the primary deuterium kinetic isotope effect. These two effects turn out to be purely additive. That is, the overall rate of reaction with deuterated DHAP at η/η° = 2.8 is 12-fold slower than the rate of protonated DHAP at η/η° = 1.0. This is the result of a 2-fold reduction in rate from the increase in solvent viscosity and a 6-fold reduction in rate due to deuterium substitution of substrate DHAP. This additivity eliminates the possibility that the two effects are due to two independent transition states approximately equal in height. The solvent-dependent motion of the protein and the enolization process are, therefore, tightly coupled.

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