Contribution of Phosphate Intrinsic Binding Energy to the Enzymatic Rate Acceleration for Triosephosphate Isomerase

Tina L. Amyes, AnnMarie C. O'Donoghue, and John P. Richard*

Department of Chemistry, University at Buffalo SUNY, Buffalo, New York 14260-3000

Received August 2, 2001

We report that 80% of the enzymatic rate acceleration for the prototypical proton transfer from carbon catalyzed by triosephosphate isomerase can be directly attributed to the remote phosphodianion group of the substrate (*R*)-glyceraldehyde 3-phosphate, and that the intrinsic binding energy of this functional group in the transition state for enzyme-catalyzed enolization is 14 kcal/mol.

Triosephosphate isomerase (TIM) catalyzes the reversible stereospecific aldose−ketose isomerization of (*R*)-glyceraldehyde 3-phosphate (GAP) to dihydroxyacetone phosphate (DHAP) by a single-base (Glu-165) proton-transfer mechanism through a *cis*-enediol(ate) intermediate (Scheme 1).[1−5] TIM is a paradigm for enzymatic catalysis of proton transfer at carbon, and catalyzes isomerization of the free carbonyl form of GAP to DHAP at the diffusion-controlled limit with $k_{cat}/K_m = 2.4 \times 10^8$ M^{-1} s^{-1}.[2,3] We consider here the possibility that the large enzymatic rate acceleration for TIM[6] is due mainly to utilization of the intrinsic binding energy of the *nonreacting* phosphate group of the substrate.[7]

Electrostatic interaction of the substrate phosphodianion with TIM is essential for optimal substrate binding and enzymatic activity.[8] For example, the K12M active site mutation drastically decreases the ability of TIM to bind both DHAP and its monoanionic analogue dihydroxyacetone sulfate.[8b] However, there has been no quantification of the contribution of the substrate phosphodianion group to the enzymatic rate acceleration. The activity of TIM toward isomerization of (*R*)-glyceraldehyde (GA) to give dihydroxyacetone (DHA) would not be detected by conventional enzyme assays, but we have found that the very slow reaction of GA in the presence of a large amount of TIM can be monitored by ^{1}H NMR spectroscopy at 500 MHz. Figure 1 (●) shows the timecourse for the disappearance of 90% of 11 mM GA in the presence of 0.17 mM (4.5 mg/mL) rabbit muscle TIM and 24 mM imidazole buffer (pD 7.0) in D_2O at 25 °C and $I = 0.1$ (NaCl).[9] ^{1}H NMR analysis showed that GA exists as 94.6% hydrate and 5.4% free carbonyl under these conditions ($K_{hyd} = 17.5$) and the reaction was followed by monitoring the disappearance of the C-1 proton of GA hydrate at 4.84 ppm (d, $J = 5$ Hz).[11] The data give $k_{obsd} = 4.6 \times 10^{-6}$ s^{-1} for disappearance of GA, which is 50-fold larger than $k_{obsd} = 9.0 \times 10^{-8}$ s^{-1} determined in the absence of TIM under the same conditions. Essentially all of the observed reaction of GA can be attributed to the presence of the protein catalyst with $k_{enz} = 4.5 \times 10^{-6}$ s^{-1}. Product analysis by ^{1}H NMR showed that ca. 60% of the products correspond to those of isomerization to give both H-DHA and D-DHA and deuterium exchange into substrate to give D-GA (Scheme 2). These product data will be discussed in a full report.

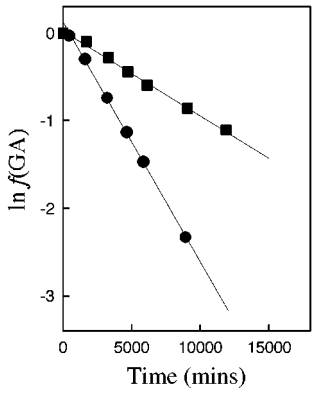
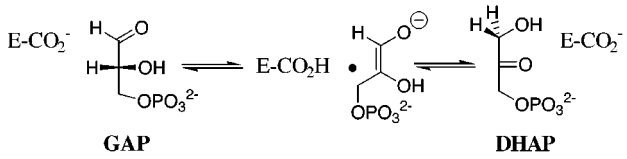


Figure 1. Logarithmic timecourses for the disappearance of 11 mM (*R*)-glyceraldehyde catalyzed by 0.17 mM rabbit muscle TIM at pD 7.0 in D_2O at 25 °C and $I = 0.1$ (NaCl). Values of k_{obsd} (s^{-1}) were obtained from the least-squares slopes (solid lines). Key: (●) In the presence of 24 mM imidazole buffer. (■) In the presence of 18 mM imidazole buffer and 8 mM 2-phosphoglycolate ($K_i \approx 5$ μM).[12,13]

Scheme 1



Scheme 2

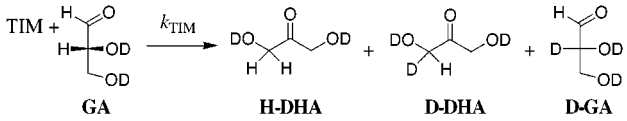


Figure 1 (■) shows the timecourse for the disappearance of 11 mM GA in the presence of 0.17 mM (4.5 mg/mL) rabbit muscle TIM, 18 mM imidazole buffer (pD 7.0), and 8 mM of the potent competitive inhibitor 2-phosphoglycolate (PGA, $K_i \approx 5$ μM)[12,13] in D_2O at 25 °C and $I = 0.1$ (NaCl). The data give $k_{obsd} = 1.6 \times 10^{-6}$ s^{-1} for disappearance of GA, which is 10-fold larger than $k_{obsd} = 1.6 \times 10^{-7}$ s^{-1} determined in the absence of

* Address correspondence to this author: (phone) (716) 645 6800 ext 2194; (fax) (716) 645 6963; (e-mail) jrichard@chem.buffalo.edu.

(1) Rieder, S. V.; Rose, I. A. *J. Biol. Chem.* **1959**, *234*, 1007−1010. Rose, I. A. *Brookhaven Symp. Biol.* **1962**, *15*, 293−309. Noltmann, E. A. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1972; Vol. VI, pp 272−354.

(2) Putman, S. J.; Coulson, A. F. W.; Farley, I. R. T.; Riddleston, B.; Knowles, J. R. *Biochem. J.* **1972**, *129*, 301−310. Plaut, B.; Knowles, J. R. *Biochem. J.* **1972**, *129*, 311−320.

(3) Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5627−5631. Knowles, J. R.; Albery, W. J. *Acc. Chem. Res.* **1977**, *10*, 105−111.

(4) Lolis, E.; Petsko, G. A. *Biochemistry* **1990**, *29*, 6619−6625.

(5) Harris, T. K.; Abeygunawardana, C.; Mildvan, A. S. *Biochemistry* **1997**, *36*, 14661−14675.

(6) Richard, J. P. *J. Am. Chem. Soc.* **1984**, *106*, 4926−4936.

(7) Jencks, W. P. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1975**, 43, 219−410.

(8) (a) Belasco, J. G.; Herlihy, J. M.; Knowles, J. R. *Biochemistry* **1978**, *17*, 2971−2978. (b) Lodi, P. J.; Chang, L. C.; Knowles, J. R.; Komives, E. A. *Biochemistry* **1994**, *33*, 2809−2814. Joseph-McCarthy, D.; Lolis, E.; Komives, E. A.; Petsko, G. A. *Biochemistry* **1994**, *33*, 2815−2823.

(9) (*R*)-Glyceraldehyde (80−90 wt % in H_2O) from Aldrich was exchanged with D_2O before use. Rabbit Muscle TIM (lyophilized powder) from Sigma was exhaustively dialyzed in the buffer of interest in D_2O. The concentration of TIM was determined from V_{max} obtained in standard assays[15] using a value of $k_{cat} = 2.6 \times 10^5$ min^{-1} for GAP.[2,10] Periodic assay[15] of the reaction mixture (after 50 000-fold dilution) for TIM activity showed that there was no significant loss of enzyme activity during the time period of these experiments (up to one week).

(10) Krietsch, W. K. G.; Pentchev, P. G.; Klingenbuerg, H.; Hofstaetter, T.; Buecher, T. *Eur. J. Biochem.* **1970**, *14*, 289−300.

(11) ^{1}H NMR spectra at 500 MHz (64 transients) were acquired at 25 °C using a Varian Unity Inova spectrometer with a 90° pulse angle, 6000 Hz sweep width, 4 s acquisition time, and a 120 s relaxation delay.

(12) Wolfenden, R. *Nature* **1969**, *223*, 704−705.

(13) Hartman, F. C.; LaMuraglia, G. M.; Tomozawa, Y.; Wolfenden, R. *Biochemistry* **1975**, *14*, 5274−5279.

10.1021/ja016754a CCC: $20.00
Published on Web 10/17/2001

TIM under the same conditions, so that $k_{enz} = 1.5 \times 10^{-6}$ s^{-1} in the presence of saturating PGA. Therefore, the isomerization of GA at the active site of TIM is *so slow* that its rate is comparable with that of nonspecific protein-catalyzed degradation of GA that occurs outside the enzyme active site. ^{1}H NMR analysis showed that only 2% of the products of the reaction of GA in the presence of TIM and saturating PGA correspond to those of isomerization to give DHA.[14] The net first-order rate constant for reaction of GA at the active site of triosephosphate isomerase can be calculated as $k_{TIM} = (4.5-1.5) \times 10^{-6} = 3.0 \times 10^{-6}$ s^{-1} (Scheme 2).

A value of $K_i = (K_m)_{GA} = 15$ mM for competitive inhibition of TIM by the *free carbonyl* form of (*R*)-glyceraldehyde can be calculated from the observed decreases in V_{max} upon addition of 64 mM total GA in competitive inhibition experiments in H_2O with GAP as substrate of 10% at $[S] = (K_m)_{GAP}$ and 20% at $[S] = 0.4(K_m)_{GAP}$.[15] This tentative value of $(K_m)_{GA} = 15$ mM for GA is consistent with the observation of good first-order kinetics for the turnover of 11 mM GA (0.6 mM free carbonyl form) by TIM (Figure 1), which requires $(K_m)_{GA} \gg 0.6$ mM for free GA in D_2O. The second-order rate constant for deprotonation of (*R*)-glyceraldehyde by TIM in D_2O at pD 7.0 can be calculated from eq 1 as $k_{cat}/K_m = 0.34 \pm 0.05$ M^{-1} s^{-1} (average value from two experiments).[16]

$$k_{cat} / K_m = k_{TIM}(1 + K_{hyd}) /[E] \qquad (1)$$

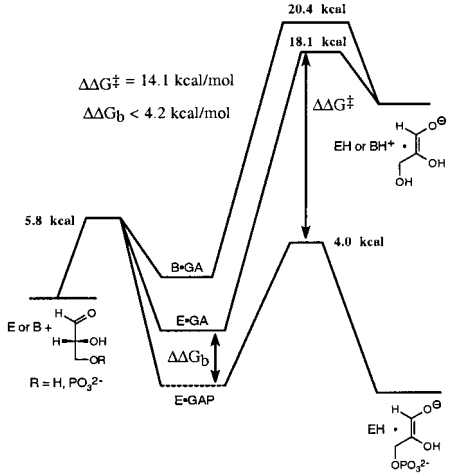


Figure 2. Free energy reaction coordinate profiles for enolization of GA (R = H, this work) and GAP (R = PO_3^{2-}, Knowles and co-workers[2,3,17]) catalyzed by TIM and of GA catalyzed by 3-quinuclidinone (B, this work). The standard state is 1 M at 298 K.

Figure 2 shows free energy reaction coordinate profiles for proton transfer from C-2 of GAP (lower profile, data of Knowles and co-workers[2,3,17]) and GA (middle profile, data from this work in D_2O)[16] to TIM. This shows that the total intrinsic binding energy of the phosphate group of GAP in the transition state for proton transfer to TIM to give the enediol(ate) is $\Delta\Delta G^{\ddagger} = 14.1$ kcal/mol. The difference in the free energies of binding of GA and GAP to TIM is $\Delta\Delta G_b < 4.2$ kcal/mol (Figure 2), which is close to the free energy of binding of inorganic phosphate to the enzyme ($K_i \approx 5$ mM at pH 7.7).[12] Therefore, more than 70% (> 10 kcal/mol) of the total intrinsic binding energy of the phosphate group of GAP is "utilized" to increase the rate constant for proton transfer from the enzyme-bound carbon acid by at least 10^7-fold.[7]

The value of $k_{cat}/K_m = 0.34$ M^{-1} s^{-1} for proton transfer from C-2 of GA to triosephosphate isomerase is only 50-fold larger than the second-order rate constant $k_B = 6.5 \times 10^{-3}$ M^{-1} s^{-1} for deprotonation of GA by the small general base catalyst 3-quinuclidinone ($pK_a = 7.5$) (Figure 2, upper profile).[18] The *specific* activity of TIM (FW 26 000)[10] is 4-fold *smaller* than that of 3-quinuclidinone (FW 125)! The *total* rate acceleration for triosephosphate isomerase, calculated as the ratio of the second-order rate constants for deprotonation of GAP by TIM and 3-quinuclidinone, is given by $[(k_{cat}/K_m)/k_B] = 2.4 \times 10^8/6.5 \times 10^{-3} = 4 \times 10^{10}$.[2,6,19] Therefore, our data show that ca. 84% of the enzymatic rate acceleration for TIM can be *directly* attributed to utilization of the intrinsic binding energy of the small substrate phosphodianion group that is remote from the site of chemical transformation.[7]

There is a large binding interaction of >17 kcal/mol for the ribose 5′-phosphate group of orotidine 5′-phosphate in the transition state for the reaction catalyzed by orotidine 5′-phosphate decarboxylase.[20] To the best of our knowledge, the present work is the first demonstration that the binding energy of a single small functional group at a substrate accounts for almost the entire enzymatic rate acceleration. This highlights the importance of charged groups such as phosphodianions in providing binding energy that can be utilized for catalysis. Sugar isomerases catalyze the isomerization of sugars lacking the charged phosphate group with the assistance of a highly charged metal dication,[21] but are far less efficient than sugar phosphate isomerases.[22,23]

In summary, attempts to understand the rate acceleration for TIM should focus on elucidation of the physical mechanism for the differential expression of binding interactions of the protein, particularly the essential residues of the flexible "loop"[24] and the active site Lys-12[8b] with the substrate phosphodianion group at both the ground-state Michaelis complex and the transition state for enzyme-catalyzed deprotonation of triosephosphates.[7]

Acknowledgment. We acknowledge National Institutes of Health Grant GM 39754 for generous support of this work.

JA016754A

(14) The major product of reaction of GA in the presence of TIM and saturating PGA is methylglyoxal. By analogy with protein glycation by glyceraldehyde [Glomb, M. A.; Monnier, V. M. *J. Biol. Chem.* **1995**, *270*, 10017−10026] this likely results from elimination of water at a protein−glyceraldehyde Schiff's base adduct [Thornalley, P. J.; Langborg, A.; Minhas, H. S. *Biochem. J.* **1999**, *344*, 109−116].

(15) Assays of TIM with GAP as substrate were carried out in 0.10 M triethanolamine buffer (pH 7.5) at 25 °C and $I = 0.1$ (NaCl).[2] A value of $(K_m)_{GAP} = 0.45$ mM for GAP for rabbit muscle TIM was determined in our experiments and is in good agreement with that for chicken muscle TIM at 30 °C at the same pH and ionic strength.[2]

(16) Experiments at $I = 0.1$ (NaCl) showed that there is no change in $(K_m)_{GAP}$ and a <2-fold decrease in k_{cat} for GAP in imidazole buffer (pD 7.0) in D_2O compared with triethanolamine buffer (pH 7.5) in H_2O.

(17) There is no significant difference in k_{cat}[10] or $(K_m)_{GAP}$ (this work) for GAP with rabbit muscle TIM at 25 °C and chicken muscle TIM at 30 °C.[2] Therefore the data for chicken TIM at 30 °C[2] were used in Figure 2.

(18) k_B was determined by monitoring the initial velocity of formation of D-DHA (up to 10%) from 10 mM GA in the presence of 63−180 mM 3-quinuclindinone (pD 7.28) in D_2O at 25 °C and $I = 1.0$ (KCl) by ^{1}H NMR. There was negligible formation of H-DHA and D-GA, which provides further strong evidence that their formation from GA in the presence of TIM results from reaction at the active site of the isomerase.

(19) We use $k_B = 6.5 \times 10^{-3}$ M^{-1} s^{-1} for deprotonation of both GA and GAP by 3-quinuclidinone at 25 °C because it is very similar to $k_B = 4.2 \times 10^{-2}$ M^{-1} s^{-1} for deprotonation of GAP in H_2O at 37 °C.[6]

(20) Miller, B. G.; Snider, M. J.; Short, S. A.; Wolfenden, R. *Biochemistry* **2000**, *39*, 8113−8118.

(21) Allen, K. N.; Lavie, A.; Glasfeld, A.; Tanada, T. N.; Gerrity, D. P.; Carlson, S. C.; Farber, G. K.; Petsko, G. A.; Ringe, D. *Biochemistry* **1994**, *33*, 1488−1494.

(22) Farber, G. K.; Glasfeld, A.; Tiraby, G.; Ringe, D.; Petsko, G. A. *Biochemistry* **1989**, *28*, 7289−7297.

(23) Nagorski, R. W.; Richard, J. P. *J. Am. Chem. Soc.* **2001**, *123*, 794−802.

(24) Pompliano, D. L.; Peyman, A.; Knowles, J. R. *Biochemistry* **1990**, *29*, 3186−3194. Xiang, J.; Sun, J.; Sampson, N. S. *J. Mol. Biol.* **2001**, *307*, 1103−1112.