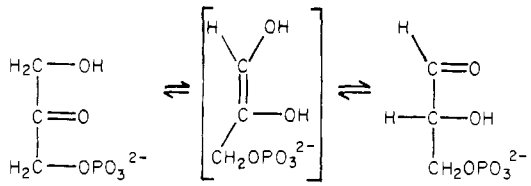
Partition of Intermediates of Triosephosphate Isomerase: Slow Conformational Changes Precede Enolization and Follow Product Release†

Irwin A. Rose* and Radha Iyengar

ABSTRACT: The relative absolute values of the apparently simple first-order reactions of triosephosphate isomerase have been determined by isotope partition measurements of two kinds. (1) The partition of the central E·enediol-P intermediate to form free dihydroxyacetone-P and glyceraldehyde-3-P was measured in tritiated water. The ratio of initial labeling was independent of the direction of chemical flow, thereby establishing that there is only one kinetically distinguishable enediol-P intermediate at which $^3H^+$ enters the reaction. (2) The partition of E·glyceraldehyde-3-P into free products was determined by diluting concentrated solutions of enzyme·substrate complexes into a medium containing ~0.2 M phosphoglycolate, a strong competitive inhibitor, followed by trichloroacetic acid to prevent further turnovers of the enzyme. This technique makes use of the fact that glyceraldehyde-3-P present in the concentrated enzyme solution represents ~55% of the total population of enzyme complexes compared with <5% present as free glyceraldehyde-3-P at solution equilibrium. This partition to immediate products together with the partition of E·enediol-P and E·dihydroxyacetone-P intermediates allows an assignment of rate constants that is consistent with the ratio of the steady-state V_{max} values. The partition, measured as the ratio of dihydroxyacetone-P to glyceraldehyde-3-P formed, was significantly greater for the E·enediol-P complex than for E·glyceraldehyde-3-P. This was especially true in D_2O. However, the fact that tritium from water always enters glyceraldehyde-3-P without discrimination seems to require that the two complexes are in rapid equilibrium. To accommodate the observations made by the two kinds of partition experiments requires that there be a slow conformational change of E·glyceraldehyde-3-P formed from the intermediate to a second form that then liberates the product. The absence of a D_2O effect in the cycle of catalysis requires that the real rate-limiting step is external to the reaction process in the recycling of free enzyme from a product-derived form to a substrate-utilizing form. Comparison is made of the isomerases of rabbit and chicken muscle, yeast, and *Bacillus stearothermophilus* with respect to the partition of E·glyceraldehyde-3-P to free products. Large differences in kinetic constants are indicated despite conservation of the equilibria between the major enzyme·ligand forms.

Triosephosphate isomerase (EC 5.3.1.1) is a widely distributed enzyme that catalyzes the interconversion of dihydroxyacetone-P (DHAP), and glyceraldehyde-3-P (G3P), through the tightly bound enediol-P intermediate (E·X).



The enzyme, M_r ~50000, has two identical subunits, does not require a cofactor for its function, uses only the carbonyl forms as substrates, and is approximately diffusion controlled in the direction G3P → DHAP, $V_{max}/K_m = 3.7 \times 10^8$ M s^{-1} (Albery & Knowles, 1976a). Characterization by amino acid sequence, crystal structure, and useful kinetic studies makes this least complex of the aldose–ketose isomerases an important object for further study both to learn more about the chemistry of enzyme-catalyzed enolizations and to define the changes in structure that the enzymes use to lower the energy barrier for the chemical interconversion. The present paper is drawn to the conclusion that conformational changes, not necessarily involved in the reaction step, are important in determining the rate of the chemical reaction.

The point of departure for these studies is the recent observation by Iyengar & Rose (1981a,b) with triose-P isomerase at equilibrium with its substrates that the concentrations of the three enzyme-bound chemical species dihydroxyacetone-P (E·DH), D-glyceraldehyde-3-P (E·GH), and the enediol-3-P intermediate (E·X) are in the ratio 0.41:0.54:0.05. These values, originally established with the rabbit muscle enzyme, have since been found for the isomerases of yeast, chicken muscle, and *Bacillus stearothermophilus*. Prospects for determining all of the rate constants and correlating kinetic differences with amino acid sequence differences between enzymes are a foreseeable goal with these triose-P isomerases for which much structural, kinetic, and mechanistic data already exist. For this to be done, the extensive studies using chicken muscle isomerase by Knowles & Albery (1977), in which some of the rate relationships appropriate to the generally accepted mechanism abbreviated in Scheme I were elaborated, must be completed and refined. However, conventional kinetic methods do not resolve internal steps (except when observed isotope effects can be compared with established intrinsic values), and the two V_{max} and V_{max}/K_m values are insufficient to establish any rate constants for a mechanism such as this. Therefore, the earlier studies could not have been expected to establish individual rate constants accurately. The very significant difference between the equilibrium ratio of enzyme-bound substrates, E·DH/E·GH = 0.76, and the corrected solution ratio of ~420 (Reynolds et al., 1971) suggested a further kinetic approach based on determining the distribution of triose phosphates immediately following dilution of the equilibrium mixture of enzyme-bound complexes. The partition of bound into free substrates would depend on the interconversion rate of bound forms relative to the rates of their release, i.e., all the first-order steps of the catalytic pathway of Scheme I. With these data and the additional value for

† From the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111. *Received October 2, 1981.* This work was supported by U.S. Public Health Service Grants GM-20940, CA-06927, and RR-05539 and also by an appropriation from the Commonwealth of Pennsylvania.

0006-2960/82/0421-1591$01.25/0

Scheme I

$$DH + E \underset{2}{\overset{1}{\rightleftharpoons}} E{\cdot}DH \underset{4}{\overset{3}{\rightleftharpoons}} E{\cdot}X \underset{6}{\overset{5}{\rightleftharpoons}} E{\cdot}GH \underset{8}{\overset{7}{\rightleftharpoons}} E + GH$$

the partition of the E·X intermediate to free products, it was possible to determine the *relative* magnitude of all first-order rate constants (k_2–k_7, Scheme I). In addition, from partition studies in D_2O it seems likely that the simple scheme is in need of expansion to include kinetically important steps.

Materials and Methods

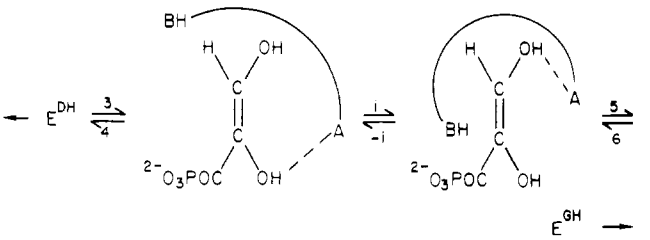
Rabbit muscle triosephosphate isomerase was obtained from Boehringer Mannheim and was used without further purification. Chicken muscle isomerase was a generous gift from Dr. J. R. Knowles, and the enzyme of *B. stearothermophilus* was prepared by Dr. J. Ieuan Harris. Protein concentration was measured from the A_{280} value by assuming the absorbance of 1 mg of pure isomerase/mL to be 1.21/cm and the weight of the catalytic subunit to be 26 000 and 25 000 daltons for the rabbit and chicken isomerases, respectively (McVittie et al., 1972). Triosephosphate isomerase was assayed with DL-G3P as the substrate and α-glycerophosphate dehydrogenase as the coupling enzyme. α-Glycerophosphate dehydrogenase was purchased from Boehringer and was treated with glycidol phosphate (Rose & O'Connell, 1969) to inactivate contaminating triosephosphate isomerase. Other enzymes were obtained as follows: yeast isomerase, muscle aldolase, and glycerol kinase (*Escherichia coli*) were from Sigma Chemical Co.; α-glycerophosphate dehydrogenase (rabbit muscle) and glyceraldehyde-P dehydrogenase (rabbit muscle) were from Boehringer.

^{32}P-Labeled dihydroxyacetone phosphate ([^{32}P]DHAP) was prepared as described previously (Iyengar & Rose, 1981a). It was further purified by high-pressure liquid chromatography (HPLC) on a Varian 5000 instrument by using an anion-exchange column and sodium acetate (0.6 M, pH 4.5) as the eluting buffer. The preparation was free of G3P but contained ~1% of the total radioactivity as $^{32}P_i$. The specific activity of the [^{32}P]DHAP was determined by the change in the NADH absorbance at 340 nm upon treatment with α-glycerol-P dehydrogenase and the decrease in alkaline-labile $^{32}P_i$ produced thereby. [5-^{3}H]Glucose (Amersham), containing a small amount of ^{3}H in other positions, was used to make fructose 1,6-bisphosphate (fructose-1,6-P_2) to evaluate the transfer of ^{3}H from G3P to DHAP, as will be described later.

Update Instrument Inc. System 1000 was used for the rapid quench experiments. In the usual experiment, the pulse solution containing triose-P isomerase and [^{32}P]DHAP in 20 mM sodium cacodylate, pH 6.5, was injected from the line that connected a syringe (loaded with 0.5 mL of H_2O) to the four-grid mixer that received 4 volumes of 0.3 M phosphoglycolate in 20 mM cacodylate from a 2-mL syringe. The solution aged as it travelled to a second mixer where it was joined by 4 volumes of 0.3 N trichloroacetic acid (Cl_3CCOOH) from a second 2-mL syringe, thus inactivating the enzyme. The flow time between the mixers was varied from 8 to 20 ms. By loading samples into the connecting tubing instead of into syringes, it was possible to work with small volumes such as 20 μL without leaving a major fraction in the tubing and mixer after the push.

Assays for ^{32}P in each triose-P were based on the ability of α-glycerol-P dehydrogenase to prevent conversion of DHAP to P_i in alkali and of isomerase plus glycerol-P dehydrogenase to stabilize the ^{32}P of G3P (Iyengar & Rose, 1981a). $^{32}P_i$ was determined by counting the isobutyl alcohol phase after separation from an acid ammonium molybdate solution (Berenblum & Chain, 1938). Because the yeast enzyme was not fully inactivated by Cl_3CCOOH, it was found necessary to prevent reactivation by incubating the acidified reaction mixture with pepsin (Rose & O'Connell, 1969) prior to neutralization and analysis of the triose phosphates. Except where noted, all experiments were done at 23 °C with the enzyme from rabbit muscle.

Scheme II



Results

Partition of E·X. Scheme I makes no mention of at least one demonstrable step. It does not include the side reaction responsible for the exchange, with rate constant k_x, of the proton being transferred with the proton of the medium. This has been concluded to be very rapid by Rieder & Rose (1958), who found negligible ^{3}H transfer from DHAP to G3P with rabbit enzyme, and by Herlihy et al. (1976), who found ~6% transfer. A further possibility that should be considered that makes use of this exchange property is whether there are *two* forms of E·X, each formed immediately from one or the other substrate as shown in Scheme II. If both E·X forms are capable of proton exchange with the medium and if they are not interconverted more rapidly than they return to their sources, Scheme II predicts that the ratio of tritium from ^{3}HOH that initially enters the two triose phosphates will depend on the direction of the net flux. Tritium entering G3P should be greater relative to ^{3}H entering DHAP when G3P is used as the substrate and DHAP is trapped. Identical exchange/conversion incorporation ratios obtained in the two directions would signify that only one E·enediol-P intermediate capable of exchange could be distinguished.

Knowles and co-workers (Maister et al., 1976; Fletcher et al., 1976) have already carried out exchange/conversion measurements with chicken muscle isomerase, but the partitions in the two directions were evaluated in separate publications, and although very similar values were obtained, they should be determined at the same time with the intended purpose of comparison. The partition of ^{3}HOH into DHAP and G3P was therefore measured after only 10% substrate consumption with assured complete trapping by α-glycerol-P dehydrogenase or glyceraldehyde-P dehydrogenase. The latter coupling, especially, needs to be very effective in view of the ~400 times higher V_{max}/K_m value for G3P required by the Haldane relationship. As shown in Table I, the partition of tritium into DHAP vs. G3P was no greater when DHAP was the substrate than when it was the product. Therefore, any rearrangements of conformation, hydrogen bonding, or solvent distribution that may occur between two E·enediol-P species are too rapid to be distinguished. Only a single intermediate can be recognized by partition methods.

From the isotope discrimination factors observed (Table I), the partition of E·X to the *protonated* products favors DHAP formation by ~3.4-fold, differing significantly from the 8-fold ratio of V_{max} values observed under the same conditions. The 3.4 value is independent of the extent of direct transfer of ^{1}H between the substrates which, of course, lowers the incorporation of $^3H^+$ from the medium and so makes the apparent

Table I: Partition of E·X in ^3HOH or $^3H^+$ in D_2O [a]

expt	reaction [h]	product formed: nmol	product formed: ^3H (cpm)	app $H/^3H$ effect [b]	substrate (cpm) [c]	E·X partition [d]: D^3H/G^3H	E·X partition [d]: DH/GH
(A) rabbit enzyme in $H_2O(^3H)$							
1	DHAP $\xrightarrow[(548)]{}$ G3P	94	35790	1.44	35520	0.99	3.27
2	G3P $\xrightarrow[(265)]{}$ DHAP	107	5929	4.78	5488	1.08	3.55
						av:	3.36
(B) rabbit enzyme in $D_2O(^3H)$							
3	G3P $\xrightarrow[(109)]{}$ DHAP	208	7306	3.1	7076	1.03	3.19 [e]
(C) chicken enzyme in $H_2O(^3H)$							
4	G3P $\xrightarrow[(106)]{}$ DHAP	103	2748	3.97	3084	0.89	2.72 [g]
5 [f]	G3P $\xrightarrow[(101)]{}$ DHAP	103	2604	3.99	2900	0.90	2.75 [g]

[a] (A) In going to G3P, 0.3 mL of ^3HOH contained [^{32}P]DHAP (940 nmol), NAD^+ (0.66 mM), $NaAsO_4$ (2 mM), EDTA (3.3 mM), DTT (4 mM), sodium pyruvate (0.65 mM), D-glyceraldehyde-3-P dehydrogenase (4 IU), lactate dehydrogenase (4 IU), rabbit or chicken isomerase (10^{-3} IU), and sodium cacodylate (20 mM, pH 6.5). When Cl_3CCOOH was added to terminate the reaction, the loss of pyruvate relative to a control indicated the amount of product formed. In going to DHAP, 0.3 mL of ^3H in H_2O or D_2O contained sodium cacodylate (20 mM, pH 6.5), G3P (1000 nmol or 2000 nmol with D_2O), NADH (0.66 mM), glycerol-P dehydrogenase (4 IU), and rabbit or chicken isomerase (10^{-3} IU, except in experiment 5 where 10^{-2} IU was added with 2 mM phosphoglycolate). [^{32}P]Glycerol-3-P and [^{32}P]glycerate-3-P were added as internal standards for recovery on the Dowex 1-Cl^- ion-exchange column. (B) and (C) were set up by using the same format as used above, always consuming ~10% of the D-G3P present initially. [b] Specific activity of product/specific activity of ^3H_2O. [c] Corrected for substrate ultilization by increasing the observed counts by 5%. [d] This ratio is independent of the extent of direct proton transfer. [e] Assumes a $^2H/^3H$ discrimination value of ~1.0 in going to G3P because of the large effect of D_2O in lowering $^3H^+$ discrimination toward DHAP. [f] With 2 mM phosphoglycolate and 10^{-2} IU of enzyme. [g] Using the discrimination value of 1.3 determined by Maister et al. (1976). [h] Numbers in parentheses on arrows are specific activities of the water in cpm/nanoatom.

discrimination against ^3H too high.

A similar experiment that will be referred to again was done under the same conditions from G3P → DHAP in D_2O that contained ^3H, pD = 6.9 (Table IB). The discrimination between ^3H and ^2H was 3.10, slightly less than that between ^3H and ^1H, but the partition of $^3H^+$ was about the same in D_2O, and the partition corrected for ^3H discrimination was somewhat lower, 3.2 compared with 3.4. The discrimination in forming [^3H]G3P in D_2O may be expected to be less than the 1.4 found in H_2O and is neglected in this calculation. Also shown is an experment with chicken muscle isomerase (Table IC) where the ^3H partition and $^1H/^3H$ discrimination in forming DHAP are similar to the values for rabbit. The discrimination value is 3-fold lower than the value found by Fletcher et al. (1976) although the partition of ^1H is about the same. This suggests the possibility of a strong pH effect on k_3 relative to k_2 in that the earlier study was done at pH 7.6 compared to 6.5 used here. Phosphoglycolate at ~200 times its K_i had no effect on the partition of E·X.

Partition of E·DH and E·GH. A mixture of [^{32}P]DHAP and excess isomerase sufficient to bind both triose phosphates completely was either quenched directly with Cl_3CCOOH, giving the distribution of enzyme-bound species, or mixed with a solution of the competitive inhibitor phosphoglycolate, 0.3 M, pH 6.5, and quenched with acid at measured times thereafter. From the high catalytic rate of the enzyme, >500 s^{-1}, it is expected that the ^{32}P-labeled components initially bound to the enzyme will have completed their partition to free forms within 1 ms of mixing with the phosphoglycolate. This inhibitor is meant to suppress the reaction of *free* enzyme with the liberated substrates and allow time to bring in the Cl_3CCOOH. The effectiveness of the inhibitor in allowing the initial partition to occur with little subsequent reaction may be judged from Figure 1A,C with rabbit and chicken isomerases. The points fall on lines of shallow slope that extrapolate to much lower amounts of [^{32}P]G3P than expected for a true zero time. The difference represents the partition of E·G3P that occurred in the first instant of mixing. Because the K_i of phosphoglycolate increases as the pH is increased (Hartman et al., 1975), it was important to keep the chase solution at pH 6.5 which begins the long plateau of the maximum velocity that extends to pH 10 (Plant & Knowles, 1972).

To calculate rate constants from the data of Figure 1, it is necessary to know the contribution that each of the bound species makes to the free products. The partition of the intermediates expressed as the ratio G3P/DHAP must be in the order E·GH > E·X > E·DH. The E·X value sets an upper limit of 1/4.36 or ~23% of the total E·DH that could produce free G3P. However, the true value is likely to be much less than this from the fact that $\vec{V}_{max}$, or V_{max} from G3P → DHAP, is 8–10 times greater than in the direction from DHAP where the steady-state concentration of E·DH at saturation with DHAP will be at its highest. Since

$$\overleftarrow{V}_{max} = k_2(E{\cdot}DH)^{\rightarrow}_{ss}$$

$$\vec{V}_{max} = \vec{k}_{E\cdot DH}(E{\cdot}DH)^{\rightarrow}_{ss}$$

and

$$(E{\cdot}DH)^{\rightarrow}_{ss} < (E{\cdot}DH)^{\leftarrow}_{ss}$$

k_2 must be more than 8-fold greater than the flux of E·DH to G3P, $\vec{k}_{E\cdot DH}$.

More direct evidence that E·DH partitions almost exclusively to free DHAP is obtained by using tritiated water in the pulse. As seen from Table II, the total tritium content of DHAP liberated in the dilution process is no less and is in fact significantly greater in one experiment than that of the E·DH present in the pulse. It is shown with [2-^{3}H]G3P (Table III) that 97.5% of the tritium that passes through E·X is lost by exchange for a proton before reaching DHAP, meaning that it exchanges 39 times faster than it is transferred, $k_x = 39k_4{}^3H$. The value of the discrimination against ^3H from ^3HOH (Table I) can then be used to calculate two important values: the amount of ^1H that is directly transferred from G3P to DHAP, and the isotope effect in k_4 corrected for the fact that because of direct ^1H transfer less tritium was incorporated into DHAP from ^3HOH than could be attributed to isotope discrimination. If y = the ratio of tritium to hydrogen in-

Table II: Partition of E·D^{3}H and E·G^{3}H[a]

		DHAP			G3P		
expt	quenching mode	% ^{32}P	^{3}H/^{32}P	^{3}H (cpm) (calcd)[b]	% ^{32}P	^{3}H/^{32}P	^{3}H (cpm) (calcd)
1	Cl_3CCOOH	41	0.71	3816	54	0.75	5310
	phosphoglycolate	80	0.38	3830[b]	20	0.662	1736
	recovered (%)	195		100	37	88	33
2	Cl_3CCOOH	41	5.54	13628	54	5.82	18850
	phosphoglycolate	80	3.84	18430[b]	20	5.05	6060
	recovered (%)	195		135	37	87	32

[a] Conditions were as in Figure 1 with 105 μL of pulse solution in ^3H_2O (50 mCi), [^{32}P]DHAP (11 nmol, 1.3 × 10^4 cpm), isomerase (39 nmol), and sodium cacodylate (20 mM) in syringe 1. Quenching was either direct with Cl_3CCOOH or 8 ms after mixing with 4 volumes of 300 mM glycolate-2-P. Carrier DHAP and G3P were added and converted to α-glycerol-P and glycerate-3-P, which were isolated by ion-exchange chromatography, and analyzed for ^{3}H and ^{32}P. The ^{3}H/^{32}P ratio was determined by counting the aqueous phase after removal of $^{32}P_i$ by isobutyl alcohol extraction of the molybdate complex. Experiment 2 differed only in the amounts of ^{32}P and ^{3}H in the protocol. [b] Corrected for ^{3}H expected to be incorporated with a 4.78 discrimination (Table I) into DHAP formed during the chase after the 5-fold dilution of the ^{3}HOH.

Table III: Transfer of [2-^{3}H]G3P to [1-^{3}H]DHAP[a]

	incubation	
	A	B
^{3}H in water after isomerase action on G3P → DHAP (cpm × 10^5)	447000	410000
% of total	76	83
^{3}H in water after isomerase action on DHAP from glycerol-P[b] (cpm)	11592	923
^{3}H transferred, % of the isomerase-activated counts	2.25	0.22

[a] Fructose-1,6-P_2 (2 × 10^4 cpm/nmol, ~30 nmol) with ~85% of the ^{3}H at C-5 was incubated in sodium cacodylate (20 mM, pH 6.5) buffer with rabbit muscle aldolase (0.64 IU), rabbit muscle isomerase (0.009 IU), α-glycerol-P dehydrogenase, and NADH in incubation A until reaction was complete, ~60 min. Sample B was incubated without dehydrogenase for 60 min, sufficient to exchange into water almost all of the isomerase-sensitive tritium, and α-glycerol-P dehydrogenase was then added to convert the triose phosphates to glycerol-P. ^3H_2O was recovered from each sample by lyophilization. [^{32}P]-α-Glycerol-P was added as a reference for recovery of the nonvolatile residues, and the glycerol-P was isolated on Dowex 1-Cl. The ^{3}H present in the "isomerase positions" of the glycerol-P was determined as counts made volatile by an incubation containing α-glycerol-P dehydrogenase (4.4 IU), NAD^+ (0.75 mM), and isomerase (1.9 IU). [b] These values are corrected for complete recovery.

Table IV: Effect of the pH of the Pulse on the Partition[a]

pH	chase in phosphoglycolate etc. (%)
5.5	21.3
6.5	19.4
7.5	20.3
8.5	20.6

[a] The normal partition experiment described in Figure 1 was repeated with an 8-ms delay after dilution in phosphoglycolate except that the pH of the pulse solution was varied with the following buffers: pH 5.5 and 6.5, 20 mM sodium cacodylate; pH 7.5 and 8.5, 100 mM triethanolamine hydrochloride.

Table V: H_2O/D_2O Effects on the Partition of E·GH(^{2}H)

pulse medium	pulse E·GH(^{2}H)[a]	chase medium	chase G3P[b]	D_2O effects[c] in pulse	in chase
H_2O	53.8	H_2O	19.94		
		D_2O	19.00		0.93
D_2O	52.9	H_2O	32.3	2.65	
		D_2O	30.7	2.54	0.89

[a] [^{32}P]G3P found when Cl_3CCOOH is added to the pulse solution. [b] [^{32}P]G3P found 8 ms after being mixed with 4 volumes of 0.3 M phosphoglycolate, as in Figure 1, in either H_2O or D_2O. [c] Calculated by comparing the partition E·GH → DH/E·GH → GH obtained in H_2O or D_2O where the D_2O is present in either the pulse or the chase solutions. The D_2O effect represents ratio in H_2O/ratio in D_2O.

corporated from the medium in forming DHAP and x = the fraction of direct ^{1}H transfer from G3P to DHAP, then $yx = 0.025$, the fraction of tritium transferred directly, and $y(1 - x) = 1/4.8$, the fraction of tritium incorporated by exchange. Solving these equations, we find the extent of ^{1}H transfer is 10.7%, and considering that $k_2 > k_3$ so that $k_{EX \to DH} \simeq k_4$, we conclude that $k_4/k_4^{3H} = 4.3$ (not 4.7) and that $k_x = 9k_4$.

Calculation of Rate Constant Ratios. Assuming that none of E·DH contributes to G3P in the experiments of Figure 1 and that only 1% or 1 out of 4.7 of the 5% present as E·X goes to G3P, one can specify the partition of E·GH between G3P and DHAP in terms of k_6, k_7, and the fractions of E·X, $\vec{f}_{E\cdot X}$ and $\bar{f}_{E\cdot X}$, that partition to G3P and DHAP, respectively (eq 1).

$$\vec{f}_{E\cdot GH}/\bar{f}_{E\cdot GH} = (k_7 + k_6\vec{f}_{E\cdot X})/(k_6\bar{f}_{E\cdot X}) \quad (1)$$

From Table I $\bar{f}_{E\cdot X} = 0.77$ and $\vec{f}_{E\cdot X} = 0.23$ for rabbit enzyme, and from Figure 1 the partition of E·GH is (23 − 1)/(53 −22) = 0.71. Therefore, $k_6/k_7 = 3.2$.

From these values and knowing $k_2 > k_3$, one can calculate k_4/k_5: From Table I $\bar{f}_{E\cdot X} = 3.36\vec{f}_{E\cdot X}$. Therefore, $k_2k_4/k_2 + k_3 \simeq k_4 = 3.36k_5/(1 + k_6/k_7)$ and $k_4/k_5 = 0.80$. From this and the equilibrium constants (E·GH/E·X = 10.8 and E·DH/E·X = 8.2), the additional ratios are calculated as $k_5/k_7 = 34.6$, $k_4/k_7 = 27.6$, $k_3/k_7 = 3.4$, and $k_3/k_6 = 1.1$.

D_2O Effect in Partitioning E·GH. According to Figure 1, there is a considerable effect of having D_2O in the pulse on the partition of E·GH. Here each enzyme-bound substrate of the pulse will have had its enolizable hydrogen replaced by ^{2}H, but also some of the protons on the enzyme will have been exchanged prior to being mixed with the phosphoglycolate-containing chase solution. Using the value extrapolated by line B of Figure 1 for the partition from a D_2O pulse into an H_2O chase (5-fold dilution), we found that E·G^{2}H goes to G3P 2.1 times more readily than to DHAP compared with 0.71 for E·GH.

One source of a D_2O effect would be from a change in the pK_a of a critical group on the enzyme in the D_2O pulse.

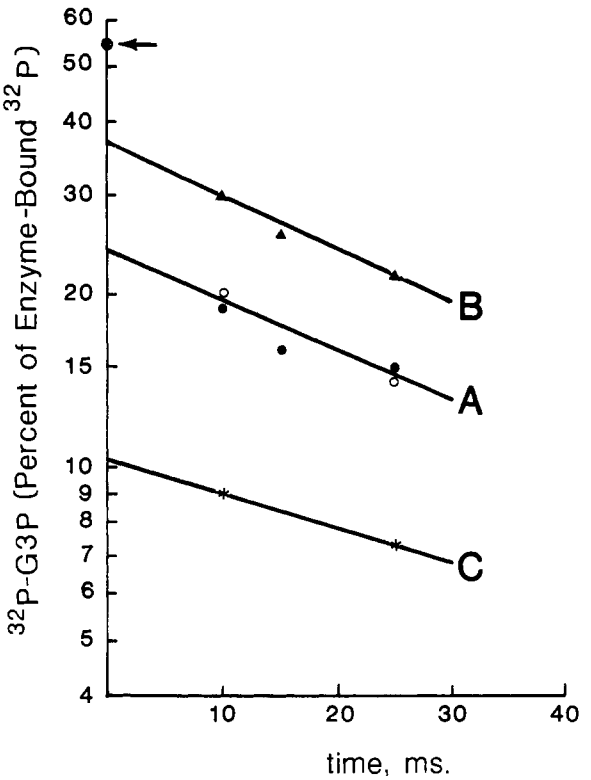


FIGURE 1: Partition of enzyme-bound species. 50 μL containing [^{32}P]DHAP (5 nmol, 1.2 × 10^4 cpm/nmol) and triosephosphate isomerase (25 nmol) from rabbit muscle in H_2O (●, ○) or D_2O (▲) or from chicken muscle (×) in sodium cacodylate (0.02 M, pH 6.5) from syringe 1 was mixed with 1 mL of glycolate-2-P (0.2 M) from syringe 2. After the aging times noted, the effluent of the mixer was quenched with Cl_3CCOOH (0.2 N final concentration). After neutralization, NADH and α-glycerol-P dehydrogenase alone or with triose-P isomerase were added followed by NaOH (0.5 N final concentration). $^{32}P_i$ resulting from alkaline decomposition of the remaining triose phosphates was determined by isobutyl alcohol extraction of its molybdate complex. In all cases, the percent of total ^{32}P present as E·GH before dilution was 53% (←). $^{32}P_2$ contaminating the starting [^{32}P]DHAP has been subtracted.

Despite the fact that the chase solution was always pH 6.5, there would be <1 ms during the partition for adjustment to the pH of 6.5 used for the chase. However, varying the pH of the pulse in H_2O from 5.5 to 8.5, while always using pH 6.5 for the diluting solution, had no effect on the partition of E·GH (Table IV).

It is not likely that the D_2O effect was due to D_2O itself because as shown in Table V the same partition was obtained whether dilution was made in H_2O or D_2O; i.e., the partition reflects the *medium* of the pulse but not its pH.

Comparison of Data for Isomerases of Several Sources. As noted from Figure 1, the partition of E·GH to G3P is relatively less favorable for isomerase from chicken although the partition of E·X as judged by the $^3H^+$ partition is slightly more favorable (Table I). When the discrimination against ^3H is corrected for, the partition of E·X is embarrassingly more favorable in the formation of G3P than is E·GH, ~0.31/1 vs. ~0.20/1. It would seem from this that in the case of the chicken enzyme there is very little direct dissociation of E·GH, that is, $k_6 \gg k_7$.

The partition of E·GH when E is yeast isomerase or the enzyme of *B. stearothermophilus* determined in Table VI can be compared with the values for the other enzymes. The partitions for chicken, bacteria, yeast, and rabbit had the 10-ms values of 0.18, 0.22, 0.35, and 0.56, which is an unusual order in evolutionary terms. Only chicken and rabbit isomerases were compared for the partition of E·X, and there they agreed.

Table VI: Partition of E·GH on the Enzyme of Yeast and *B. stearothermophilus*[a]

enzyme	quenching mode	% of ^{32}P		E·GH → G3P
		G3P	DHAP	E·GH → DHAP
yeast	Cl_3CCOOH	52.5	40.2	
	phospho-glycolate	14.6	83.3	0.35
Bacillus	Cl_3CCOOH	54.6	38.5	
	phospho-glycolate	11.0	86.9	0.25

[a] Each isomerase (38 nmol) with 10 nmol of [^{32}P]DHAP in 100 μL was chased with 0.56 mL of 0.3 M phosphoglycolate and quenched with Cl_3CCOOH at 10 ms.

All of the enzymes gave virtually identical equilibrium concentrations of intermediate and substrate complexes in spite of the difference in the partitions of E·GH.

Discussion

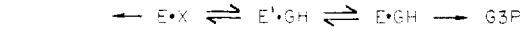
The very small discrimination against tritium in ^3HOH (Table I), also noted with chicken isomerase by Maister et al. (1976), is best explained in Scheme I if k_6 is much greater than k_7, i.e., slow product release. This is because ketonizations are expected to show a high primary isotope effect. The spontaneous ketonization of enolpyruvate in D_2O is ~6 times slower that in H_2O (Kuo & Rose, 1979). Pyruvate kinase enolizes pyruvate with a similarly large primary effect (Robinson & Rose, 1972), and muscle aldolase can show a discrimination of 23-fold against $^3H^+$ in the formation of DHAP from fructose-1,6-P (Rose et al., 1965). Therefore, discrimination against ^3H in water by isomerase the ~3-fold in forming DHAP is not likely to represent the primary isotope effect of the ketonization step, and the conclusion that $k_2 > k_3$ in Scheme I offers two alternatives, either the ketonization has an unusual mechanism or the step is complex and includes a slow conformation change that is independent of proton transfer.

There seems no doubt of an enolization mechanism (Iyengar & Rose, 1981b). The requirement for a slow conformational change between E·GH and E·X is especially implied not only because there is little discrimination against ^3H of water (Table I) but also because the partition of E·GH(^3H) did not result in ^3H enrichment of the G3P formed (Table II), and the product-release step is not slow enough, $k_7 = 0.31k_6$, to obscure a large primary isotope effect in k_5.

The effect of D_2O in favor of dissociation to G3P is too large to be attributed to a primary effect in the reaction itself as judged from the values for tritium (Table II). The D_2O effect on k_6 was calculated to be 2.1 whereas the tritium content and specific activity of G3P derived from E·GH(^3H) are consistent with $k_6/k_6^{3H} = 1.2$. If the difference in the partition observed in D_2O had been due to a primary effect arising from cleavage of the C–^2H bond of bound G3P, one predicts that the effect of tritium, which as a tracer can only express a primary effect, would be even larger: $2.1^{1.44} = 2.9$ according to the Swain relationship. This would lead to a 2-fold increase in the tritium specific activity of the G3P instead of a 12% decrease. Therefore, almost all of the effect of D_2O is unrelated to enolization chemistry. D_2O effects of the order of 1.3–1.5 are commonly observed in general acid- and base-catalyzed enolizations (Reitz & Kopp, 1939; Long & Watson, 1958). However, these "solvent effects" are always prequilibria to the –CH bond cleavage so that large primary effects are always found.

The D_2O effect on the partition of E·GH, being independent of the medium used in the dilution, must be a consequence

Scheme III



of deuterium that has replaced protons from exchangeable positions such as –NH, –OH, –SH, etc. Many of these would have exchanged during the several minutes that the pulse solution was being manipulated for the experiment, but the exchanged ^2H atoms would not have time to exchange with the medium in the <1-ms time that the enzyme takes to partition its products. These deuterium atoms in many of the hydrogen bonds of the protein could influence the rate of a conformational change, such as proposed above, required for the enolization of E·GH.

Therefore, two additional species should be added to Scheme I. These are E′·DH and E′·GH. More can be surmised about E′·GH (Scheme III) which would be described as follows: the conformational change E′·GH → E·GH is much slower than the reversal to E·X; hence, no discrimination is seen in forming G3P in ^3HOH. The release of E·GH is still the slowest step in the forward direction, being ~0.3 times the rate of reversal to E′·GH for the rabbit enzyme and less than that for the isomerase from chicken.

In the consideration of substrate-dependent conformational changes in chicken and rabbit isomerases, the work of Waley and co-workers using UV difference spectroscopy (Jones & Waley, 1979) and exchange characteristics of protein-bound protons (Browne & Waley, 1979) should be considered. Browne and Waley observed significant differences between competitive inhibitors such as α-glycerol phosphate and phosphoglycolate both present at saturation in their effects on the exchange rates of tritium. Substrates were particularly effective, causing an almost 10-fold increase in the half-life of more than 200 hydrogens. Differences between enzymes from rabbit and chicken isomerases were shown that might be related to the differences seen in the partition of E·GH.

As noted already, the equilibrium distribution of intermediates did not differ significantly in spite of rather large extents of inhomology in amino acid sequences of enzymes from lower and higher forms. Alber et al. (1981) find that the conformations of the polypeptide chain of chicken and yeast isomerases are essentially identical in the crystal state. Thus, the forces that stabilize the equilibria may include gross structural interactions conserved in evolution as well as local interactions involving the substrates or intermediates.

One would like to be able to go further and use the ratios of rate constants that have been determined and the steady-state V_{max} values to calculate the absolute values of the rate constants for k_2–k_7 according to Scheme I. To do this, one must be assured that a rate-determining step does not occur in the rearrangement of free enzyme. Only in that case can the expression for k_{cat} depend only on k_2–k_7. Indeed, when this assumption is made for the reaction, the absolute values that are calculated are in reasonable agreement when the forward and reverse directions are considered. However, from the partition measurements, the rate-determining step for the conversion of E·GH to E + DHAP must be the conformational change E·GH → E′·GH. If this step has an isotope effect of ~2 in D_2O, one should expect that $\vec{V}_{max}$ would also be slower by a factor of ~2. In fact, one observes no D_2O effect on any of the steady-state parameters at either pH 6.5 or pH 7.5. These results do not constitute a contradiction, but rather they emphasize a distinction between partition and turnover measurements. For example, a slow conformational change occurring in the free enzyme after release of product will not influence a partition ratio but may be rate determining in overall catalytic turnover. Indeed, such an extra step with a rate constant of $\sim 10^4$ s^{-1} seems to be indicated by the absence of a D_2O effect in the rapid direction of catalysis, G3P to DHAP. This step must be insensitive to D_2O.

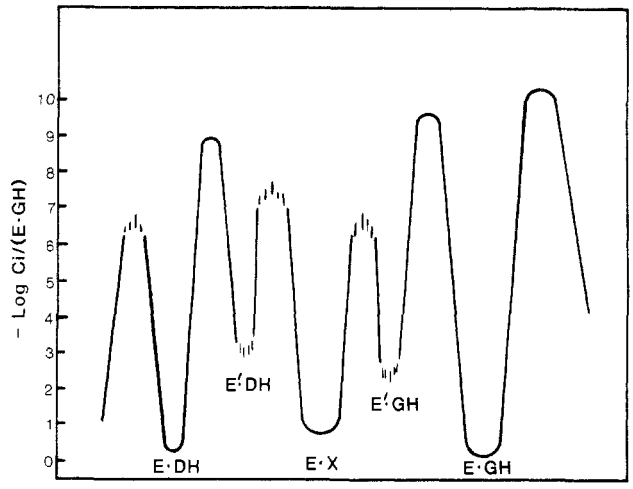


FIGURE 2: Energy diagram showing concentrations of enzyme-bound species and transition states [calculated from $K^{\ddagger} = hk/(\kappa T)$ relative to E·GH]. The magnitude of k_7 (~1250 s^{-1}) was estimated from $\vec{V}_{max}$ by assuming that external steps do not contribute significantly in the slower forward direction, $\vec{V}/E_T$ = 650 s^{-1}, pH 6.5, 23 °C.

The distribution of enzyme among the forms E·DH/E·X/E·GH in the steady state can be calculated from the appropriate "net rate constants" according to Cleland (1975). Using the ratio of rate constants relative to k_7 for rabbit isomerase at pH 6.5 ($k_2/k_3/k_4/k_5/k_6/k_7$ = >3.4/3.4/27.6/34.6/3.2/1), one calculates the three forms to be in the ratio 1.4/0.12/1 in the forward direction and ~0/0.033/1 in the reverse direction. These may be compared with the distribution at equilibrium of 0.79/0.09/1. Figure 2 summarizes in an "energy diagram" the first-order relationships responsible for this equilibrium, taking note also of the additional forms E′·GH and E′·DH.

References

Alber, T., Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D., Rivers, P. S., & Wilson, I. A. (1981) *Philos. Trans. R. Soc. London, Ser. B 293*, 159–171.

Albery, W. J., & Knowles, J. R. (1976a) *Biochemistry 15*, 5627–5631.

Albery, W. J., & Knowles, J. R. (1976b) *Biochemistry 15*, 5631–5640.

Berenblum, I., & Chain, E. (1938) *Biochem. J. 32*, 295.

Browne, C. A., & Waley, S. G. (1974) *Biochem. J. 141*, 753–760.

Cleland, W. W. (1975) *Biochemistry 14*, 3220–3224.

Fletcher, S. J., Herlihy, J. M., Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5612–5617.

Hartman, F. C., Le Muraglia, G. M., Tomozawa, Y., & Wolfenden, R. (1975) *Biochemistry 14*, 5274–5279.

Herlihy, J. M., Maister, S. G., Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5601–5607.

Iyengar, R., & Rose, I. A. (1981a) *Biochemistry 20*, 1229–1235.

Iyengar, R., & Rose, I. A. (1981b) *Biochemistry 20*, 1223–1229.

Jones, R. B., & Waley, S. G. (1979) *Biochem. J. 179*, 623–630.

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

Kuo, D., & Rose, I. A. (1979) *J. Am. Chem. Soc. 101*, 5025–5030.

Long, F. A., & Watson, D. (1958) *J. Chem. Soc.*, 2019–2024.
Maister, S. G., Pett, C. P., Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5607–5611.
McVittie, J. D., Esnouf, M. P., & Peacocke, A. R. (1972) *Eur. J. Biochem. 29*, 67–73.
Plaut, B., & Knowles, J. R. (1972) *Biochem. J. 129*, 311–320.
Reitz, O., & Kopp, J. (1939) *Z. Phys. Chem., Abt. A 184*, 429.
Reynolds, S. J., Yates, D. W., & Pogson, C. I. (1971) *Biochem. J. 122*, 285–297.
Rieder, S. V., & Rose, I. A. (1959) *J. Biol. Chem. 234*, 1007–1010.
Robinson, J., & Rose, I. A. (1972) *J. Biol. Chem. 247*, 1096–1105.
Rose, I. A., & O'Connell, E. L. (1969) *J. Biol. Chem. 244*, 126–134.
Rose, I. A., & O'Connell, E. L. (1977) *J. Biol. Chem. 252*, 479–482.
Rose, I. A., O'Connell, E. L., & Mehler, A. (1965) *J. Biol. Chem. 240*, 1758–1765.

Specificity of the Catalytic Interaction of Human DNA Polymerase β with Nucleic Acid Substrates†

Teresa Shu-Fong Wang and David Korn*

ABSTRACT: We have employed steady-state kinetics methodology together with novel assays of the polymerization products synthesized on DNA primer-templates of known sequence to obtain new insights into the mechanism of catalysis of human DNA polymerase β. A major objective of these studies has been to define structural elements of DNA substrates that are required for catalytically productive polymerase binding. The results demonstrate very substantial differences between KB cell polymerases α and β with respect to the molecular signals that govern their specific catalytic interactions with nucleic acids. We show that substrate addition to polymerase β obeys a rigidly ordered sequential mechanism, with DNA first followed by dNTP. Under reaction conditions optimized for gapped (activated) DNA primer-template, with Mg^{2+} or Mn^{2+} as the divalent cation, polymerase β exhibits no detectable kinetic affinity for intact duplex DNA molecules, whether covalently closed circles or blunt-ended linear fragments, and both natural and synthetic, linear and circular single-stranded polydeoxynucleotides produce patterns of inhibition that are, under all conditions tested, fully and linearly noncompetitive with DNA (and dNTP) substrates. Polymerase β has a very high affinity for duplex DNA molecules that contain nicks bearing either 3′-OH or 3′-PO_4 termini, and 3′-PO_4-terminated nicked DNA is a potent inhibitor of the polymerase β reaction that is linearly competitive with DNA substrate. From a detailed examination of the interaction of polymerase β with staggered-end duplex DNA molecules of known sequence, we present strong evidence in support of the conclusion that a primary determinant of the productive binding of the polymerase to DNA is a base-paired primer moiety that must be adjacent to a very short length of (potentially) single-stranded template. Our inability kinetically to resolve separate partial reactions of primer binding and template binding suggests that in contrast to KB cell polymerase α, primer-template binding of polymerase β may occur by a concerted mechanism. The choice of divalent cation has a dramatic effect on the minimum length of template required for binding. Thus, in the presence of Mg^{2+}, the minimum template length is greater than or equal to five nucleotides, while in the presence of Mn^{2+} only a single template nucleotide is sufficient. Finally, we demonstrate that the choice of divalent cation also affects the processivity of deoxynucleotide incorporation by human polymerase β with DNA. With Mg^{2+} as cation, the polymerization mechanism is essentially distributive, with about one nucleotide inserted per binding cycle. In contrast, with Mn^{2+} as cation, the reaction mechanism is modestly processive, with insertion of four to six nucleotides in each polymerization cycle. Both the values of processivity and their response to the divalent cation are identical when measured on nicked and gapped DNA substrates.

The availability of essentially homogeneous preparations of human DNA polymerases α and β (Fisher & Korn, 1977; Wang et al., 1974, 1977), that are completely free of associated or contaminating deoxyribonuclease activities (Fisher et al., 1979; Wang & Korn, 1980), has permitted a detailed investigation of some of their enzymological properties with a variety of defined, natural and synthetic DNA primer-templates. These studies have demonstrated a number of striking differences between the two enzymes with respect to their ability to catalyze deoxynucleotide incorporation on these several DNA substrates (Eichler et al., 1977; Korn et al., 1978; Fisher et al., 1979; Wang & Korn, 1980) and have suggested that there might be equally profound differences, possibly of physiological significance, in the nature of the specific molecular signals that regulate the catalytic interactions of the two polymerases with nucleic acids.

In recent reports (Fisher & Korn, 1979a,b, 1981a,b; Fisher et al., 1981), we have employed steady-state kinetics methodology together with direct sedimentation binding assays to document several of the key features of the catalytic mechanism of KB cell DNA polymerase α. (1) The interaction of polymerase α with its substrates obeys a rigidly ordered sequential terreactant mechanism, with template (single-stranded polydeoxynucleotide) as the first substrate followed by primer as the second substrate and dNTP as the third. (2) Although

† From the Laboratory of Experimental Oncology, Department of Pathology, Stanford University School of Medicine, Stanford, California 94305. *Received September 17, 1981.* These studies were supported by Grant CA-14835 from the National Institutes of Health.

0006-2960/82/0421-1597$01.25/0