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Mechanism for Activation of Triosephosphate Isomerase by Phosphite Dianion: The Role of a Ligand-Driven Conformational Change

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Abstract

The L232A mutation at triosephosphate isomerase (TIM) from *Trypanosoma brucei brucei* results in a small 6-fold decrease in k_{cat}/K_m for the reversible enzyme-catalyzed isomerization of glyceraldehyde 3-phosphate to give dihydroxyacetone phosphate. By contrast, this mutation leads to a 17-fold *increase* in the second-order rate constant for the TIM-catalyzed proton transfer reaction of the truncated substrate piece [1-^{13}C]-glycolaldehyde ([1-^{13}C]-GA) in D_2O; a 25-fold *increase* in the third-order rate constant for reaction of the substrate pieces GA + and phosphite dianion (HPO_3^{2-}); and a 16-fold *decrease* in K_d for binding of HPO_3^{2-} to the free enzyme. Most significantly, the mutation also results in an 11-fold *decrease* in the extent of activation of the enzyme towards turnover of GA by bound HPO_3^{2-}. The data provide striking evidence that the L232A mutation leads to a ca. 1.7 kcal/mol stabilization of a catalytically active loop-closed form of TIM (E_c) relative to an inactive open form (E_o). We propose that this is due to the relief, at L232A mutant TIM, of unfavorable steric interactions between the bulky hydrophobic side chain of Leu-232 and the basic carboxylate side chain of Glu-167, the catalytic base, which destabilize E_c relative to E_o.

Triosephosphate isomerase (TIM) catalyzes the stereospecific and reversible 1,2-hydrogen shift at dihydroxyacetone phosphate (DHAP) to give (*R*)-glyceraldehyde 3-phosphate (GAP) by a single base (Glu-167)[1] proton transfer mechanism through an enzyme-bound cis-enediolate reaction intermediate (Scheme 1).[2-4] TIM catalyzes proton transfer at carbon in an early step in glycolysis, a remarkably successful metabolic pathway.[5,6] The enzyme appeared early during the evolution of life, and is potentially an ancestor of other enzymes that catalyze deprotonation of carbon, or that contain the eponymous TIM barrel. Conclusions about the mechanism of action of TIM might therefore be generalized to many other enzymatic reactions.

The identity and location of the catalytic amino acid side chains at TIM define the elementary chemical steps for enzyme-catalyzed isomerization.[7,8] By contrast, there is no consensus of opinion about the origin of the large catalytic rate acceleration. We have shown that TIM uses binding interactions with the nonreacting phosphodianion group of the substrate to stabilize the transition state for enzyme-catalyzed isomerization of GAP by 12 kcal/mol, which represents ca. 80% of the total enzymatic rate acceleration.[9] The binding interactions with the phosphodianion group anchor the substrates GAP and DHAP to TIM and activate the enzyme for catalysis of carbon deprotonation.[10] Around 50% of this

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ASSOCIATED CONTENT

Supporting Information. Procedure for preparation of L232A mutant *Tbb* TIM. This material is available free of charge via the Internet at http://pubs.acs.org.

intrinsic phosphate binding energy is observed as specific stabilization of the transition state by the binding of phosphite dianion to the transition state for the TIM-catalyzed reaction of the truncated substrate glycolaldehyde (GA) in a two-part substrate experiment, where the covalent connection between the carbon acid and phosphodianion parts of the substrate has been eliminated.[10,11]

Flexible loop 6 of the TIM barrel fold is disordered at unliganded TIM but is folded over the phosphodianion group of enzyme-bound DHAP[12,13] and the inhibitors 2-phosphoglycolate (PGA)[14] and 2-phosphoglycolohydroxamate (PGH).[15] This closure of loop 6 over the substrate or inhibitor (Figure 1) is the most dramatic of the many changes in the protein conformation that occur upon formation of complexes between TIM and phosphodianion ligands.[4] We have proposed that these ligand-induced conformational changes activate TIM for catalysis of deprotonation of carbon.[10,11,16,17]

Wierenga and coworkers made the astute observation that the closure of loop 6 of TIM over the bound ligand PGA results in movement of the hydrophobic side chain of Ile-172 toward the carboxylate side chain of the catalytic base Glu-167 and “drives” this anionic side chain toward the hydrophobic side chain of Leu-232, which maintains a nearly fixed position (see Figure 1).[18] This conformational change sandwiches the catalytic base at the loop-closed enzyme between two hydrophobic side chains (Figure 1) and shields it from interactions with bulk solvent. This hydrophobic local environment should lead to an increase in the basicity of the carboxylate side chain of Glu-167 and hence in its reactivity toward deprotonation of carbon, relative to its reactivity in aqueous solution.

If Leu-232 plays a significant role in activating TIM for catalysis of deprotonation of carbon, then the L232A mutation should result in significant changes in the kinetic parameters for TIM-catalyzed reactions. We prepared the L232A mutant of TIM from *Trypanosoma brucei brucei* (*Tbb* TIM) starting from a plasmid containing the gene for wildtype *Tbb* TIM,[17,19] using the standard protocol described in the Supporting Information. Table 1 gives the kinetic parameters for the isomerization reactions of GAP and DHAP catalyzed by wildtype[17] and L232A mutant *Tbb* TIM at pH 7.5, 25 °C and I = 0.1. These data show that the L232A mutation leads to only a small 6-fold falloff in k_{cat}/K_m for the enzyme-catalyzed reactions of GAP and DHAP. The mutation results in a small decrease in K_m for isomerization of GAP, but a surprisingly large 9-fold *decrease* in K_m for the reaction of DHAP, and a compensating 60-fold decrease in k_{cat} for its isomerization to give GAP (Table 1).

The disappearance of [1-^{13}C]-GA catalyzed by L232A mutant *Tbb* TIM in D_2O to give the products of proton transfer (a mixture of [2-^{13}C]-GA, [2-^{13}C, 2-^{2}H]-GA and [1-^{13}C, 2-^{2}H]-GA) at pD 7.0, 25 °C and I = 0.1 in the absence and presence of phosphite dianion was monitored by ^{1}H NMR spectroscopy, as described previously for the wildtype enzyme.[17] First-order rate constants, k_{obs} (s^{-1}), were determined from the slopes of linear semi-logarithmic plots of reaction progress against time covering 70 - 80 % of the reaction according to eq 1, where f_S is the fraction of [1-^{13}C]-GA that remains at time t. The observed second-order rate constants for these TIM-catalyzed proton transfer reactions of [1-^{13}C]-GA in D_2O, $(k_{cat}/K_m)_{obs}$ (M^{-1} s^{-1}), were determined from the values of k_{obs} using eq 2, where f_{hyd} = 0.94 is the fraction of [1-^{13}C]-GA present as the hydrate form and [E] is the concentration of TIM.[10,11] We also determined the yields of the products of the L232A *Tbb* TIM-catalyzed reactions of GAP[20] and [1-^{13}C]-GA[10,11,17] in D_2O and these data will be reported in a later publication.

$$\ln f_s = -k_{obs}t \quad (1)$$

$$(k_{cat}/K_m)_{obs} = \frac{k_{obs}}{(1 - f_{hyd})[E]} \quad (2)$$

$$\left(\frac{k_{cat}}{K_m}\right)_{obs} = \left(\frac{K_d}{K_d + [HPO_3^{2-}]}\right)\left(\frac{k_{cat}}{K_m}\right)_E + \left(\frac{[HPO_3^{2-}]}{K_d + [HPO_3^{2-}]}\right)\left(\frac{k_{cat}}{K_m}\right)_{E\bullet HPi} \quad (3)$$

Figure 2 shows the effect of increasing concentrations of phosphite dianion on $(k_{cat}/K_m)_{obs}$ for the deprotonation of [1-^{13}C]-GA catalyzed by wildtype *Tbb* TIM[17] and by the L232A mutant enzyme. These data were fit to eq 3, derived for Scheme 2, with the values of $(k_{cat}/K_m)_E$ for the unactivated reaction in the absence of phosphite (Table 1), to give the values of K_d and $(k_{cat}/K_m)_{E\bullet HPi}$ reported in Table 1. We note the following effects of the L232A mutation on these kinetic parameters: (1) A 17-fold *increase* in the second-order rate constant $(k_{cat}/K_m)_E$ for the unactivated reaction of GA from 0.07 to 1.2 M^{-1} s^{-1}. (2) A 24-fold *increase* in the third-order rate constant $(k_{cat}/K_m)_{E\bullet HPi}/K_d$ for reaction of the substrate pieces {GA + HPO_3^{2-}}. (3) A 16-fold *decrease* in the dissociation constant K_d for the phosphite dianion activator, from 19 mM to 1.2 mM. (4) Only a small change in the second-order rate constant $(k_{cat}/K_m)_{E\bullet HPi}$ for reaction of the substrate piece GA catalyzed by the TIM•HPO_3^{2-} complex, from 64 M^{-1} s^{-1} to 100 M^{-1} s^{-1}. (5) An 11-fold *decrease*, from 900-fold to 80-fold, in the *extent* of activation of *Tbb* TIM towards deprotonation of GA upon the binding of phosphite to give the E•HPO_3^{2-} complex, calculated as the ratio $(k_{cat}/K_m)_{E\bullet HPi}/(k_{cat}/K_m)_E$ (Scheme 2). In summary, to our surprise, the L232A mutation leads to only a relatively small decrease in k_{cat}/K_m for the physiological isomerization reactions of GAP and DHAP, but to an *increase* in the reactivity of the enzyme towards the substrate pieces GA and phosphite dianion in a two-part substrate experiment.

The observation that the mutation of a highly conserved residue results in an *increase* in the efficiency of the TIM-catalyzed reaction of the substrate pieces (increases in $(k_{cat}/K_m)_E$ and $(k_{cat}/K_m)_{E\bullet HPi}/K_d$) provides strong evidence that the deleted hydrophobic side chain of Leu-232 plays an important role in the activation of TIM for deprotonation of carbon. They are consistent with the proposal that the L232A mutation leads to an increase in the equilibrium constant K_c for the thermodynamically unfavorable conversion of an inactive loop open form of TIM (E_o) to a higher energy, but active, loop closed enzyme (E_c) that shows a high specificity for the binding of phosphite dianion and the transition state for deprotonation of GA (Scheme 3).[10,11,16] In this model the closed enzyme E_c is assumed to have a reactivity toward carbon deprotonation that is essentially identical to that of the phosphite-liganded closed enzyme species E_c•HPO_3^{2-}, so that $(k_{cat}/K_m)_E' = (k_{cat}/K_m)_{E\bullet HPi}$ (Scheme 3).[10,11,16] The value of K_c can then be obtained from the *ratio* of the second-order rate constants for turnover of the substrate piece GA by the phosphite-liganded enzyme and the free enzyme according to eq 5, which is the magnitude of activation of the enzyme by the binding of phosphite dianion (Table 1). The increase in K_c due to the L232A mutation is estimated to be ca. 17-fold, calculated as the average of the effects of the mutation on the kinetic parameters $(k_{cat}/K_m)_E$ (17-fold), K_d (16-fold), $(k_{cat}/K_m)_{E\bullet HPi}/K_d$ (24-fold) and the observed 11-fold decrease in the extent of enzyme activation by bound phosphite (Table 1).

$$\frac{1}{K_c} = \frac{(k_{cat}/K_m)_{E\bullet HPi}}{(k_{cat}/K_m)_E} = \frac{K_d}{K_d'} \quad (5)$$

Figure 3 illustrates the effect of a decrease in ΔG_c (increase in K_c) and hence an increase in the fraction of enzyme present in the active closed form (E_c, Scheme 3) on the kinetic parameters for reaction of the substrate pieces {GA + HPO_3^{2-}}. The overall barrier to the change from E_o to E_c (ΔG_c = 4.0 kcal/mol) for wildtype *Tbb* TIM is given by the SUM of the red and black bars in the lower left hand corner of Figure 3. The red bars show the magnitude of the effect of the L232A mutation on this barrier, $\Delta\Delta G_c \approx 1.7$ kcal/mol. The decrease in ΔG_c for L232A mutant TIM is expected to lead to the following changes in the kinetic parameters that depend on the fraction of enzyme present as E_c (Figure 3): (1) An increase in the second-order rate constant for turnover of the substrate piece GA, $(k_{cat}/K_m)_E$, as a result of stabilization of the transition state $E_c \bullet S^{\ddagger}$ relative to the ground state E_o + S. (2) A decrease in the dissociation constant for the phosphite dianion activator (K_d), due to the stabilization of $E_c \bullet HPO_3^{2-}$. (3) An increase in the third-order rate constant $(k_{cat}/K_m)_{E \bullet HPi}/K_d$ for turnover of the two-part substrate {GA + HPO_3^{2-}} as a result of stabilization of $E_c \bullet HPO_3^{2-} \bullet S^{\ddagger}$. On the other hand, the magnitude of the activation of TIM for deprotonation of GA by the binding of phosphite dianion should *decrease* with increasing K_c, until only a minimal two-fold activation is observed for K_c = 1 (eq 5). The observed 11-fold decrease in phosphite activation of the L232A mutant enzyme-catalyzed reaction of GA from 900-fold to 80-fold (Table 1) is therefore consistent with an increase in K_c (decrease in ΔG_c, Figure 3). We suggest that the effect of the L232A mutation on $\Delta\Delta G_c$ (Figure 3) results from the relief of a ca. 1.7 kcal/mol destabilizing steric interaction between the hydrophobic side chain of Leu-232 and the carboxylate anion of Glu-167, the catalytic base. We propose that this strain is induced by loop closure that moves the carboxylate side chain from a catalytically inactive swung-out position to the reactive swung-in conformation (Figure 1).[4,18]

The small ca. six-fold decreases in k_{cat}/K_m for the TIM-catalyzed isomerization of the whole substrates GAP or DHAP as a result of the L232A mutation (Table 1) cannot be rationalized simply by an increase in the concentration of active E_c relative to inactive E_o. We note that this mutation results in even larger ca. 150-fold decreases in the *ratio* of k_{cat}/K_m for isomerization of the whole substrate GAP or DHAP and $(k_{cat}/K_m)_{E \bullet HPi}/K_d$ for reaction of the two-part substrate {GA + HPO_3^{2-}}. Effective catalysis of the reaction of the whole substrate or of the pieces {GA + HPO_3^{2-}} requires the development of optimal binding interactions between TIM and the carbon acid and dianion portions of the substrate or the pieces. We suggest that the L232A mutation leads to a small shift in the position of the active site catalytic residues that leads to a specific *decrease* in the stabilization of the transition state for the reaction of the whole substrate GAP or DHAP, but not for the reaction of {GA + HPO_3^{2-}}, because these pieces are able to move independently at the active site.

The proposal that unliganded TIM exists mainly in the catalytically inactive open form E_o might appear to make TIM less perfect than an enzyme that exists exclusively in the active form, because the second-order rate constant for the reaction of poor substrates such as GA with the ground state E_o will decrease in direct proportion to the barrier to loop closing, ΔG_c (Figure 3). However, the second-order rate constant for the reaction of the physiological substrate GAP will approach the “perfect” diffusion-controlled limit, provided conversion of the first-formed $E_o \bullet$GAP complex by loop closing to give the catalytically active $E_c \bullet$GAP complex and then to product is faster than dissociation of GAP from $E_o \bullet$GAP. There is evidence that the closure of loop 6 over the substrate analog glycerol 3-phosphate is fast relative to the turnover of GAP,[21] and that release of bound GAP from TIM is slower than its conversion to DHAP.[2]

The catalytic advantage to the inclusion of an unfavorable ligand-driven conformational change from E_o to E_c is that this provides a simple mechanism to *attenuate* the expression of the very strong enzyme-phosphodianion interactions at the Michaelis complex. This is

necessary in order to avoid tight binding to TIM which could lead to slow, rate-determining, release of products.[22] The observed 9-fold decrease in K_m for the reaction of DHAP (Table 1) shows that the L232A mutation does in fact result in a larger expression of the binding interactions of DHAP at the Michaelis complex, at the expense of a decrease in k_{cat}. The *intrinsic* binding energy of the substrates GAP and DHAP that is utilized to drive an unfavorable conformational change prior to product formation will not be expressed in the kinetic parameter K_m. Rather, if the dianion-driven conformational change *activates* TIM for deprotonation of GAP then this binding energy will be expressed as stabilization of the transition state for deprotonation of bound substrate and an increase in k_{cat}. The observation that the binding of phosphite dianion strongly activates TIM for catalysis of deprotonation of the substrate piece GA suggests that these dianion interactions also activate TIM for catalysis of deprotonation of GAP and DHAP.[10,11,16]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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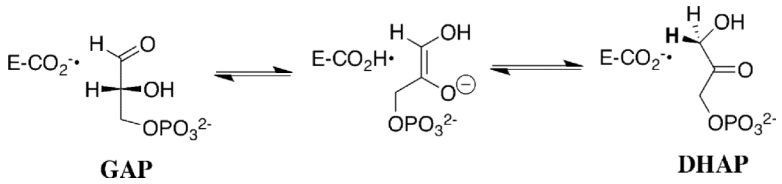


Scheme 1.

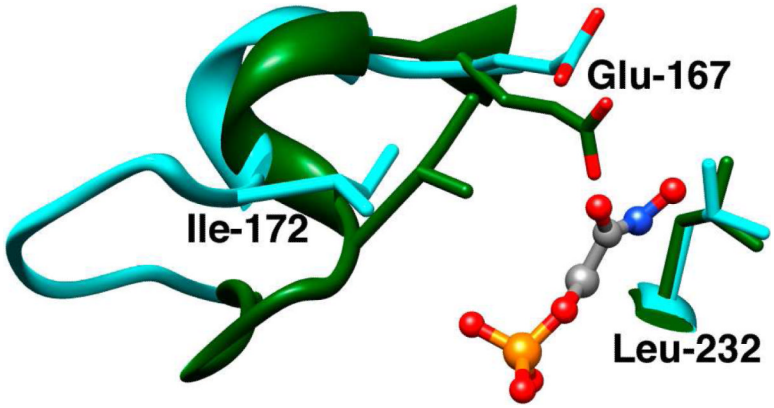
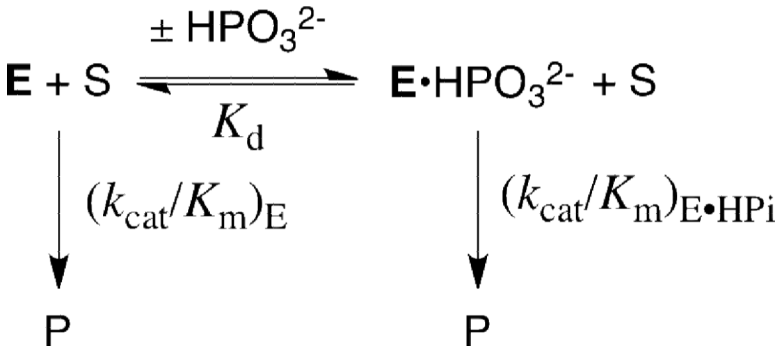


Figure 1.

Models, from X-ray crystal structures, of the unliganded open (cyan, PDB entry 5TIM) and the PGH-liganded closed (green, PDB entry 1TRD) forms of TIM from *Trypanosoma brucei brucei* in the region of the enzyme active site. Closure of loop 6 (residues 168 – 178) over the ligand phosphodianion group results in movement of the hydrophobic side chain of Ile-172 towards the carboxylate side chain of the catalytic base Glu-167. This is accompanied by movement of Glu-167 towards the hydrophobic side chain of Leu-232, which maintains a nearly fixed position.



Scheme 2.

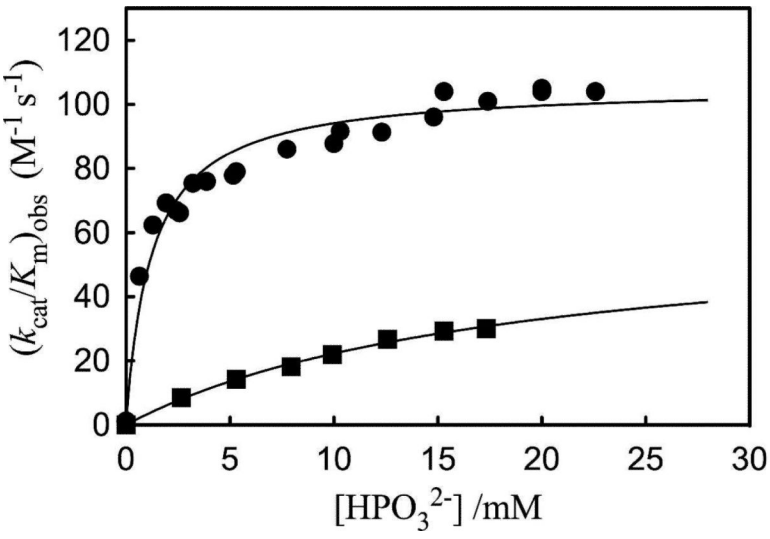
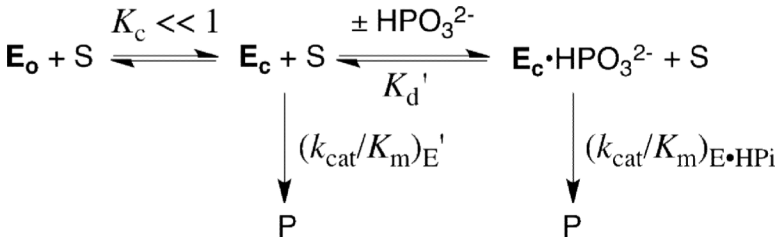


Figure 2.

The dependence of the observed second-order rate constant $(k_{cat}/K_m)_{obs}$ for the proton transfer reactions of the free carbonyl form of [1-^{13}C]-GA (20 mM total substrate) catalyzed by wildtype and L232A mutant *Tbb* TIM on the concentration of phosphite dianion in D_2O at pD 7.0 (10 - 20 mM imidazole), 25 °C and I = 0.1 (NaCl). Key: (■) Data for wildtype *Tbb* TIM (Ref. 17); (●) Data for L232A mutant *Tbb* TIM.



Scheme 3.

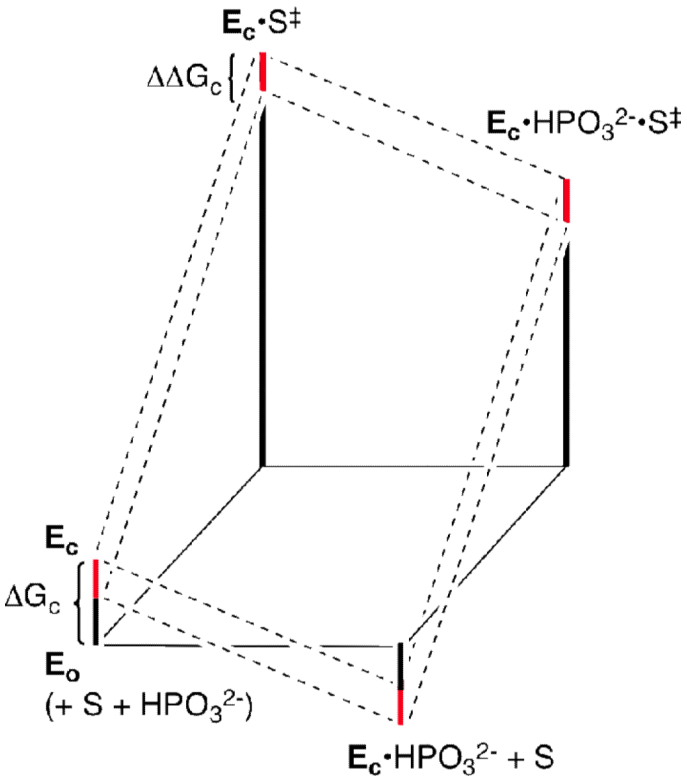


Figure 3.

Proposed free energy profiles for the turnover of glycolaldehyde (S) by free TIM (E_o) and by the phosphite-liganded enzyme $E_c \cdot HPO_3^{2-}$. The red bars show the effect of the L232A mutation on the barrier for the conformational change from E_o to E_c ($\Delta\Delta G_c$). The effect of this change in ΔG_c on turnover of the substrate pieces is shown by a comparison of the reaction profiles for wildtype TIM (upper dashed lines) and L232A mutant TIM (lower dashed lines).

Table 1

Kinetic Parameters for the reactions of GAP, DHAP, and [1-^{13}C]-GA catalyzed by wildtype and L232A mutant triosephosphate isomerase from *Trypanosoma brucei brucei*.

	GAP[a]			DHAP[a]		
***Tbb* TIM**	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m (M^{-1} s^{-1})
Wildtype[b]	2100	2.5×10^{-4}	8.4×10^6 (1.7×10^8)[c]	300	7.0×10^{-4}	4.3×10^5 (7.2×10^5)[c]
L232A	220	1.4×10^{-4}	1.5×10^6 (3.0×10^7)[c]	4.7	7.7×10^{-5}	6.1×10^4 (1.0×10^5)[c]

	[1-^{13}C]-GA[d]		{[1-^{13}C]-GA + HPO_3^{2-}}[d]		
	$(k_{cat}/K_m)_E$ (M^{-1} s^{-1})	K_d (M)	$(k_{cat}/K_m)_{E\cdot HPi}$ (M^{-1} s^{-1})	$(k_{cat}/K_m)_{E\cdot HPi}/K_d$ (M^{-2} s^{-1})	Phosphite Activation $(k_{cat}/K_m)_{E\cdot HPi}/(k_{cat}/K_m)_E$
Wildtype[b,e]	0.07	0.019	64	3400	900-fold
L232A[e]	1.2[f]	1.2×10^{-3}[g]	100[g]	8.3×10^4	80-fold

[a] At pH 7.5 (30 mM triethanolamine), 25 °C and I = 0.1 (NaCl), determined as described previously.[17] The range of error in the reported values of k_{cat} and K_m is estimated to be ± 10%.

[b] Data from Ref. 17.

[c] The values in parentheses have been corrected for the fraction of GAP (5%) or DHAP (60%) present in the reactive free carbonyl form.

[d] In D_2O at pD 7.0 (10 - 20 mM imidazole), 25 °C and I = 0.1 (NaCl). The range of error in the reported values of k_{cat}/K_m and K_d is estimated to be ± 10%.

[e] The reported parameters refer to the reactive free carbonyl form of [1-^{13}C]-GA.

[f] Average of two determinations in the absence of phosphite dianion.

[g] Determined from the fit of data in Figure 2 to eq 3.