The kinetic consequences of altering the catalytic residues of triosephosphate isomerase

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[Plate 1]

The essential basic residue at the active site of the glycolytic enzyme triosephosphate isomerase is Glu-165, which is responsible for the abstraction of either the 1-pro-R proton of dihydroxyacetone phosphate or of the 2-proton of D-glyceraldehyde 3-phosphate, in the enolization steps that constitute the reaction catalysed by this enzyme. We have changed this residue to Asp by oligonucleotide-mediated site-directed mutagenesis, and have evaluated the free-energy profile for the mutant protein. Comparison of the detailed energetics of the wild-type and mutant enzymes shows that only the transition-state free energies have been seriously affected, each of the enolization steps having been slowed by a factor of about one thousand. Evidently the movement of a catalytic group by less than 1 Å (1 Å = 10^{-1} nm = 10^{-10} m) has dramatic effects on catalysis, and the nature of these effects can provide important information about enzyme mechanism and function.

INTRODUCTION

The advances in molecular biology of the past few years have put us in the position where any amino acid in a protein can, in principle, be changed to any other. For the enzymologist, who has for decades been constrained only to investigate the consequences of altering the substrate, this opportunity to tinker with the enzyme is proving irresistible. Yet before embarking upon a major onslaught (the number of possible amino-acid changes being, of course, enormous), we must consider carefully whether any proposed change will lead to properly interpretable results and to a better understanding of catalytic mechanism. What criteria must be applied in the choice of a target system if we are to learn more about the nature of enzyme catalysis and about the relation between protein structure and function?

Our first criterion is that the three-dimensional structure of the enzyme should be known. Tempting as it may be to presume that the structural consequence of a single amino acid replacement will only be local, this is obviously dangerous, and we must be prepared to determine experimentally what structural changes have occurred. A difference electron-density map for the crystalline mutant protein at high resolution would clearly be a first step in defining any changes in the static structure of the enzyme. Secondly, the mechanism of the catalysed reaction should be known, at least at the level of the nature and sequence of reaction intermediates involved. In the absence of such data, any conclusions about the kinetic effect of an amino acid change would necessarily be uncertain. Thirdly, because our aim is to

understand the nature of the catalytic act, prior knowledge of the reaction energetics is important. We need to know, that is, which are the rate-determining transition states, and how each reaction intermediate partitions. Without this information, the effect of a mutation cannot be delineated, and any effort to interpret an altered catalytic activity will be bedevilled by the possibility that the amino acid alteration has resulted in a change in the rate-limiting step. We need, in short, to look at the enzymic systems that are well characterized in structural, mechanistic, and energetic terms. Only then shall we be in a position to evaluate the consequences of amino-acid replacement.

One of the relatively few enzymic systems that satisfy the criteria outlined above is triosephosphate isomerase. This glycolytic enzyme has been thoroughly investigated both structurally and functionally. The crystal structure of the native chicken muscle enzyme has been determined to 2.5 Å† (Banner *et al.* 1975) and that of the enzyme–substrate complex to 6.0 Å resolution (Phillips *et al.* 1977). Further, the structure of the yeast enzyme has been solved to 3.0 Å (Alber *et al.* 1985), and the complex of this protein with substrate has been studied at 3.5 Å (Alber & Petsko 1985). Results at yet higher resolution are likely to be forthcoming, and it is appropriate to emphasize the advantage of being able – for a reaction involving one substrate and one product – to study the productive complex of the enzyme with its natural substrate. Further structural details of the enzyme and of its interaction with substrate are available from nuclear magnetic resonance spectroscopy (Browne *et al.* 1975; Webb *et al.* 1977) and from work with Fourier transform infrared (Belasco & Knowles 1980). There is no doubt that any mutant isomerase that we may generate can be very fully characterized in structural terms.

From early mechanistic work by Rose (Rieder & Rose 1959; Rose 1962), and from exhaustive kinetic and chemical investigations (Albery & Knowles 1976a), the details of the isomerase-catalysed reaction (figure 1) have been rather tightly defined. The rates of
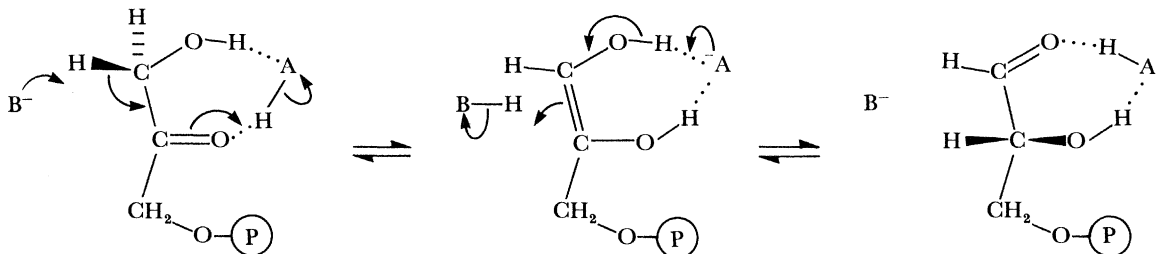

FIGURE 1. The reaction catalysed by triosephosphate isomerase. B is the enzymic base (Glu-165), and A is an enzymic acid (His-95 or Lys-13, or both).

the elementary steps have been determined, and can be summarized and illustrated as a free-energy profile (see figure 2). The facts (*a*) that the highest transition state in the free-energy profile is that involving the binding of D-glyceraldehyde-3-phosphate, which from the size of the rate constant (4×10^8 M^{-1} s^{-1}) and from viscosity variation experiments (Raines *et al.* 1985), is clearly a diffusive process, and (*b*) that at the ambient substrate level *in vivo* the dissociated reactants (enzyme plus dihydroxyacetone phosphate) are more stable than any of the reaction intermediates, mean that the isomerase has reached the end of its evolutionary development (Albery & Knowles 1976b). It is a 'perfect' catalyst, in the sense that any further acceleration

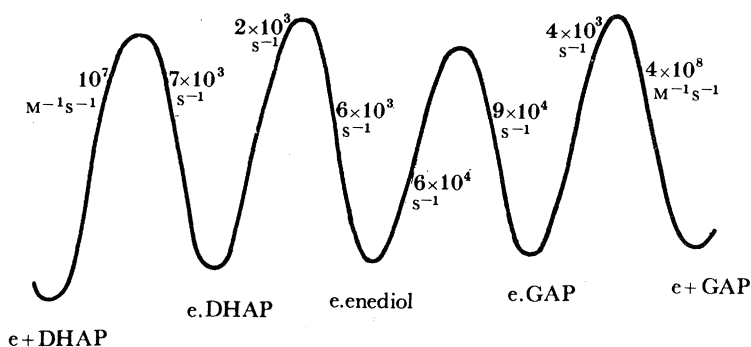<center>† 1 Å = 10^{-1} nm = 10^{-10} m.</center>

FIGURE 2. The free-energy profile for the reaction catalysed by chicken muscle triosephosphate isomerase (from Albery & Knowles 1976*a*).

of the catalytic steps (as distinct from the 'on–off' steps) would have no effect on the rate of the overall reaction.

Mechanistically, the crystallographic results, coupled with earlier chemical modification studies (Waley *et al.* 1970; de la Mare *et al.* 1972), provide some chemical clothing for the kinetic events summarized in figure 2. This is illustrated in figure 3, plate 1. The carboxylate group of Glu-165 is the base that abstracts either the 1-pro-*R*-proton of dihydroxyacetone phosphate or the 2-proton of D-glyceraldehyde phosphate. Further, the crystal structure provides two candidates (Lys-13 or His-95, or both) for the electrophile that (from both chemical (Webb & Knowles 1974, 1975) and spectroscopic (Belasco *et al.* 1980) work) evidently polarizes the substrate carbonyl group and facilitates the enolization steps. While the precise involvement of these residues must await higher resolution data on the enzyme–substrate complex, the outlines of push–pull catalysis for the two critical enolization steps, are clear. Finally, the available crystallographic results suggest that when substrate binds to the enzyme, a mobile loop of about nine residues (from 168 to 177) becomes ordered and may provide further hydrogen bonds for the binding of the substrate's phospho group (Phillips *et al.* 1977; Alber *et al.* 1981; Alber & Petsko 1985).

The above summary suggests that our knowledge of the structure and mechanism of triosephosphate isomerase make this a splendid candidate for site-directed mutagenesis. That is, the opportunity exists here for the proper evaluation of the consequences of subtle changes in critical amino acid residues, with the hope that we may yet better understand the origins and nature of the dramatic catalytic effect of this enzyme. Any manipulation of the isomerase gene requires, of course, that clones be available, and it is fortunate that the genes for the two isomerases about which most is known (those from chicken (Straus & Gilbert 1985) and from yeast (Alber & Kawasaki 1982)) have each been cloned. A cDNA (complementary) copy of the chicken muscle enzyme has been cloned, sequenced and expressed in *E. coli* (Straus & Gilbert 1985), as has the gene for the yeast enzyme (Petsko *et al.* 1984).

CHOICE OF MUTATIONS

Leaving the question of phospho-group binding and loop movement aside, it seems clear from figure 3, plate 1, that the substrate covalency changes that constitute the triosephosphate-isomerase-catalysed reaction are directly mediated by three amino-acid side chains. Glu-165

is the base, and His-95 or Lys-13 (or both) is the general acid. While there are other possibly critical residues (e.g. Glu-97, which appears to form an ion pair with Lys-13, or Ser-96, that may interact with Glu-165 and His-95), only Glu-165, His-95 and Lys-13 seem able to contact the bound substrate directly. To begin with, we have focused upon these residues, and the single alteration that is described in this paper is that of Glu-165. In the light of the rather severe catalytic consequences that are known to follow from changing the chemical nature of an essential catalytic group (e.g. the change of Ser to Cys in subtilisin (Neet & Koshland 1966; Polgar & Bender 1967), or the change of Cys to Ser in papain (Clark & Lowe 1977)), we opted to keep the *chemistry* the same, but to change the *position* of the active-site carboxylate, by making Glu-165 into Asp. While we cannot know the precise structural consequences of this change until high-resolution crystallographic data are available on the complex of mutant enzyme with substrate, it is of interest to determine the kinetic effects of moving the essential active-site base further from the substrate, by something less than 1 Å. In this way, we may hope ultimately to gain some quantitative insight into the relation between distance (from, in this case, a catalytic group to the substrate atom with which its interacts) and reaction rate.

The cDNA clone of the chicken triosephosphate isomerase gene has been placed downstream from the *trc* promoter in plasmid pX1, and the enzyme has been expressed in an *E. coli* strain (DF 502) from which the endogenous *E. coli* isomerase gene has been deleted (Straus *et al.* 1985). While DF 502 cannot grow on lactate or on glycerol as the sole carbon source (either glucose, or lactate plus glycerol, is required for DF 502 to survive), transformants of DF 502 with the plasmid carrying the chicken isomerase gene (pX1) grow normally on lactate or on glycerol alone (Straus & Gilbert 1985). When the chicken enzyme is purified to homogeneity from these transformants of *E. coli* DF 502, no kinetic differences can be detected between the enzyme produced in *E. coli* and that isolated from chicken breast muscle (Straus *et al.* 1985). The enzymes are indistinguishable on denaturing polyacrylamide gels and on isoelectric focusing, though from N-terminal sequence analysis it appears that about 20 % of the isomerase produced in *E. coli* still carries N-terminal methionine. This difference is evidently silent in kinetic terms. (Because the crystal structure shows that the N-terminus of each subunit is on the surface of the protein and some way from the active site, this result is not surprising.)

The chicken isomerase gene carried on pX1 was then used as template for oligonucleotide-mediated site-directed mutagenesis with the use of the now standard methods, to provide the mutant gene. The whole isomerase gene was then resequenced to check that the only amino acid change was Glu-165 to Asp (Straus *et al.* 1985). The plasmid containing this point mutation (ptm 2) was then used to transform the isomerase-minus *E. coli* host DF 502, and we found that transformants would grow on lactate alone, but not on glycerol alone. The level of isomerase activity in lysates of DF 502 (ptm 2) was somewhat less than 1 % of that from DF 502

DESCRIPTION OF PLATE 1

FIGURE 3. The active site of chicken triosephosphate isomerase. The coordinates are those of the native chicken enzyme (Banner *et al.* 1975) and the substrate (dihydroxyacetone phosphate) has been positioned to minimize non-bonding contacts between the enzyme and the substrate in the extended cisoid conformation (Rose 1962). The orientation of the substrate with respect to the enzyme is in accord with the abstraction of the pro-*R* proton of dihydroxyacetone phosphate by Glu-165, and the polarization of the substrate carbonyl groups by Lys-13 and His-95. The movement of the loop of residues 168–177 that is evident from the structure of the enzyme–substrate complexes (Alber *et al.* 1981; Alber & Petsko 1985) is not shown.

PLATE **1**. For description see opposite.

(pX1) (Straus *et al.* 1985). One may speculate that this lower level of isomerase, while adequate for cell survival and growth on lactate, is inadequate for growth on glycerol because the accumulated dihydroxyacetone phosphate decomposes to give lethal levels of toxic methylglyoxal (Cooper 1984). Whatever the cause of the difference, however, the cells grow well on glucose, and the mutant enzyme can be purified to homogeneity.

<div align="center">KINETIC ANALYSIS</div>

First, experiments were performed to establish that the mechanism of the reaction catalysed by the mutant isomerase was unaltered. Specifically, we were concerned to establish whether in the mutant enzyme a water molecule might interpose itself between Asp-165 and the substrate, so that while Glu-165 of the wild-type enzyme acts as a base, Asp-165 of the mutant enzyme would act as a general base. This possibility was eliminated (Raines & Knowles 1985) by studies on the solvent isotope effect and on the nature of the inactivation of the mutant enzyme with the active-site reagent, bromohydroxyacetone phosphate. The mechanisms of the mutant and wild-type enzymes are evidently identical.

As with the wild-type enzyme, the delineation of the free-energy profile of the mutant enzyme requires the combination of kinetic data from a number of different kinds of experiment. First, the steady-state kinetic parameters have been determined (Putman *et al.* 1972; Straus *et al.* 1985), and show that while the K_m values for each of the substrates are somewhat different (for the wild-type enzyme, K_m^{DHAP} is 0.97 mM and K_m^{GAP} is 0.47 mM; these quantities become 1.8 and 0.13 mM, respectively, for the mutant), the k_{cat} values are very much lower for the mutant (for the wild-type enzyme, k_{cat}^{DHAP} is 430 s^{-1} and k_{cat}^{GAP} is 4300 s^{-1}; for the mutant, these quantities are 1.8 and 2.8 s^{-1}, respectively). Secondly, several experiments which trace the fate of solvent or substrate tritium allow the partitioning of the various forms of the liganded enzyme to be evaluated. When stereospecifically labelled 1-*R*-[^{3}H]dihydroxyacetone phosphate is used as substrate for the mutant isomerase in unlabelled solvent, only a small percentage of the ^{3}H label is transferred to the 2-position of the product D-glyceraldehyde phosphate. More than 95 % of the label is lost to solvent, demonstrating that at the enediol stage of the reaction, the conjugate acid of the enzyme base (which abstracted the tritium from the pro-*R* position) rapidly exchanges its proton with the solvent. In terms of scheme 1, **A** and **B** are in rapid equilibrium with each other. This equilibration had been found earlier for the wild-type enzyme (Herlihy *et al.* 1976), and it is not surprising that the much more sluggish mutant enzyme also has time to exchange the abstracted proton at the stage of the enzyme–enediol complex.

If, now, we run the reaction of *un*labelled dihydroxyacetone phosphate in tritiated water, we may ask how the enzyme–enediol complex partitions between exchange back to substrate (that is, return to give tritiated dihydroxyacetone phosphate) and conversion forward to product (that is, reaction on to give glyceraldehyde phosphate). For the wild-type enzyme, this experiment produced the exchange–conversion graph shown in figure 4, which was interpreted as follows (Maister *et al.* 1976). At the start of the reaction, we can see from the gradient of the line that the intermediate partitions forward in the ratio of about 3:1. That is, three molecules go on to product for every molecule that returns (having picked up tritium) back to dihydroxyacetone phosphate. At later times, as tritiated substrate accumulates, we see that the specific radioactivity of the remaining starting material rises increasingly. The curvature derives from the kinetic isotope effect in the processing of 1-*R*-[^{3}H]dihydroxyacetone phosphate,

<div align="center">[83]</div>

A

B
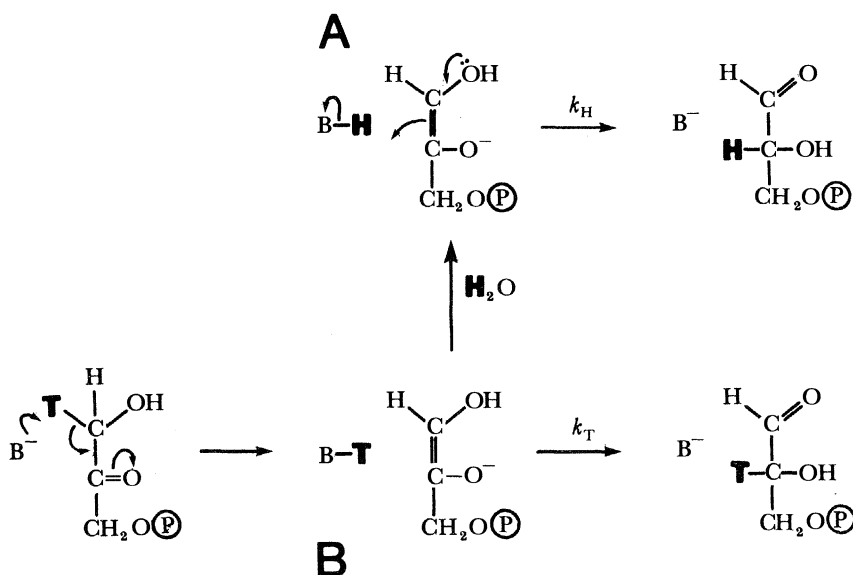
SCHEME 1. Pathway for the enzyme-catalysed reaction of $1\text{-}R\text{-}[^3H]$dihydroxyacetone phosphate.

which results in the preferential consumption of the ^1H species, leaving the ^3H molecules behind in the remaining substrate pool. When the exchange–conversion experiment is performed with the mutant enzyme, essentially no radioactivity is found in the remaining dihydroxyacetone phosphate (see figure 4). The enediol intermediate now partitions forward with a partition ratio of at least 30. Clearly the energetics of the mutant enzyme are quite different.
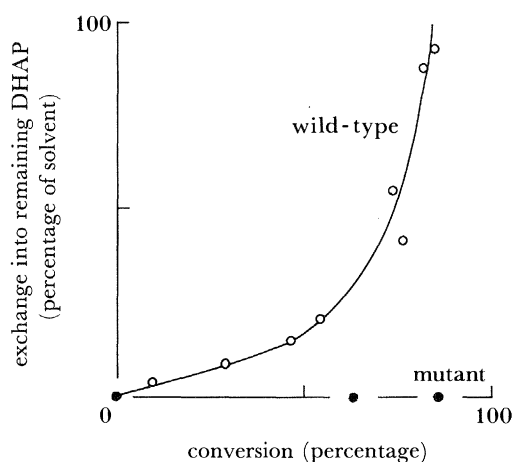

FIGURE 4. Exchange–conversion plot for wild-type chicken triosephosphate isomerase ($\circ$) and the Glu-165 to Asp mutant ($\bullet$), with dihydroxy-acetone phosphate as substrate.

Further information comes from the exchange–conversion experiment in the opposite direction, with glyceraldehyde 3-phosphate as substrate. For the wild-type enzyme, we see from figure 5 that the enediol intermediate again partitions forward in approximately 3:1 ratio (Fletcher *et al.* 1976). This ratio further defines the relative free energies of the transition states for the wild-type enzyme (for a full explanation, see Albery & Knowles 1977). For the mutant enzyme, the exchange–conversion plot is again different, and shows that the intermediate now
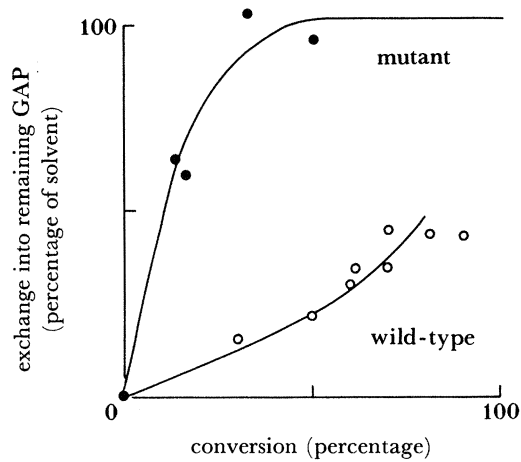
FIGURE 5. Exchange–conversion plot for wild-type chicken triosephosphate isomerase (o) and the Glu-165 to Asp mutant (●), with D-glyceraldehyde-3-phosphate as substrate.

partitions *back* to glyceraldehyde phosphate some 30 or 40 times faster than it is converted into product dihydroxyacetone phosphate (see figure 5).

The exchange–conversion results described above, coupled with the steady-state kinetic parameters and with experiments that define the discrimination against tritium in the formation of product (the results for which will be reported elsewhere) provide nearly enough data to allow the construction of the free-energy profile for the mutant enzyme. One uncertainty remains, which is the relative stability of the enzyme–enediol intermediate for the wild-type and mutant enzymes. Because this intermediate is kinetically insignificant, we must resort to an indirect approach. In the search for structural mimics of the enediol intermediate in the isomerase-catalysed reaction, both phosphoglycolate (Wolfenden 1969) and phosphoglycolo-hydroxamate (Collins 1974) were found to bind more tightly to the enzyme than either of the two substrates. Moreover, the hydroxamate has been shown by X-ray crystallography to bind at the active site in a position indistinguishable from substrate (G. A. Petsko, personal communication), and the binding constant of this molecule appears to be a fair measure of
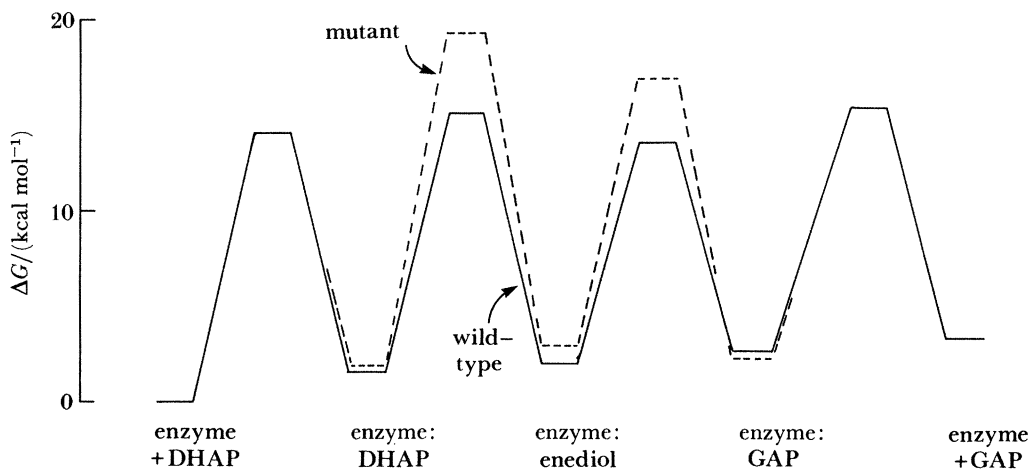

FIGURE 6. The free-energy profile for the reactions catalysed by the wild-type (solid line) and mutant (broken line) triosephosphate isomerases. (1 cal_{th} = 4.184 J.)

[85]

the interaction between enzyme and the enediol intermediate. We have therefore compared the inhibition constants for phosphoglycolohydroxamate with both the wild-type and the mutant enzymes, and find K_i values of 7.2 and 22 μM respectively (Raines & Knowles 1985). The ratio of these constants can be used – at least tentatively – to define the free energy of the enzyme–enediol intermediate.

The kinetic results described in this section can be combined and summarized in the form of a free-energy profile for the mutant enzyme, illustrated in figure 6, the implications of which are discussed below.

CATALYTIC CONSEQUENCES

As has been discussed above, our earlier evaluation of the energetics of the wild-type chicken muscle isomerase led to the conclusion that this enzyme had reached the end of its development as a catalyst (Albery & Knowles 1976 b). That is, without consideration either of possible evolutionary changes in the steady-state concentrations of the substrates in the glycolytic pathway *in vivo*, or of any benefit to cellular economics from the development of a smaller protein having the same catalytic properties, triosephosphate isomerase cannot be improved in kinetic terms. Even if this were not so, it is hardly surprising that changing the critical Glu-165 to Asp has sharply reduced the catalytic efficiency of the enzyme. At our present level of understanding of protein structure and function, most changes in most enzymes are going to be kinetically deleterious. For the isomerase, the two enolization steps in the reaction have been slowed by more than three orders of magnitude, and the overall reaction is now not limited by a diffusive transition state but by the slowed rate of the covalency changes involved in the catalytic act.

Interestingly, the free-energy levels of the bound intermediate states (substrate, product, and enediol) are relatively little changed, and it is the transition-state energies that have been raised. In terms of Pauling's view of enzyme catalysis (Pauling 1946), the binding of ground states has been changed little, but the binding of the transition states for enolization has been weakened by about 17 kJ mol^{-1} ($\approx$ 4 kcal mol^{-1}).

Whether the mutation discussed here has recreated an actual intermediate in the evolutionary development of this enzyme is doubtful, but the mutant protein does provide a basis from which we may investigate the consequences of putting *forward* evolutionary pressure on an enzyme. We can, fortunately, apply selective pressure to cells whose only isomerase is the mutant enzyme, because these transformants cannot survive on glycerol as their sole carbon source. It will be instructive to see what changes result (in both structural and kinetic terms) from the selection of second-site suppressors of our sluggish isomerase.

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