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REVIEW

Jeremy R. Knowles: The Evolution of an Enzymologist

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INTRODUCTION

"Well, naturally, my view is that it can't be a defense unless you're attacked," Jeremy informed me as I entered my oral thesis exam. Nature, however, unlike most doctoral candidates, guards her secrets tenaciously, and throughout his long and honored career, Knowles has employed the full armamentarium of the organic chemist to reveal them. His research publications, which number over 250, span the years 1960 to the present, and in each, his wit and insight are evident. At its heart, Knowles' work demonstrates that the unparalleled catalytic effectiveness of enzymes can be understood in purely chemical terms, in terms moreover of the highest sophistication. In this, of course, he has reaped from the same fertile ground first uncovered in the early fifties by Frank Westheimer, William Jencks, Thomas Bruice, Myron Bender, and Daniel Koshland. In his closing remarks at the Symposium,[1] Knowles exhorted his audience to strive "not merely to know but to understand." His own work has been the embodiment of this wise advice, and as we celebrate his 60th birthday, Knowles' influence over the field of mechanistic enzymology stands supreme. As John Albery concluded in his postprandial speech at the Saturday-night banquet, "The title of this symposium is 'Whither Enzymology,' ... but the question is 'Whence Enzymology?' and the answer is Knowles!" Here, I will review Knowles' scientific career, starting with his graduate training at Oxford and ending with his appointment as Dean at Harvard. I will dwell a bit longer upon his early work, which is perhaps less familiar to the reader, and upon triosephosphate isomerase, which has stimulated his interest for the better part of 3 decades.

In the fall of 1955, Knowles entered Balliol College at Oxford to begin his undergraduate studies. (During the previous 2 years, he had served as a Pilot Officer in the Royal Air Force, an experience that likely sharpened his instinctual ability to home in on a target and expose its inner workings.) He earned his B.A., First Class, in 1959 and then stayed on at Oxford for graduate work with Professor Richard Norman. Knowles' M.A. and D.Phil. were both awarded in 1961. His thesis

[1] "Whither Enzymology? A Symposium in Honor of Jeremy Knowles on the Occasion of His 60th Birthday," Harvard University, Cambridge, Massachusetts, April 28–30, 1995.

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work centered on electrophilic and nucleophilic aromatic substitution reactions and resulted in four publications (*1–4*), each of which reflects a firm grounding in the precepts of physical organic chemistry. The first sentence in the abstract of the first of these papers will suffice as a demonstration:

> Application of the Hammett equation to electrophilic aromatic substitution is found to be of only limited use because it fails to take into account the different extents of resonance interaction between the substituent and the aromatic system in the transition state in reactions where the reagent has different electronic requirements.

In 1960, Knowles was named to a Research Lectureship at Christ Church College, a position that allowed a 6-month leave. This he utilized in 1961 to travel to Caltech as a postdoctoral fellow.

α-CHYMOTRYPSIN AND PEPSIN

At Caltech, Knowles first worked with George Hammond, checking some theoretical calculations on the triplet levels of metal chelates. While engaged in this project, for which he has described himself as "manifestly unsuited," he began talking with Bryan Jones, who was studying the substrate specificity of α-chymotrypsin in the lab of Carl Niemann. Soon metal chelates were just a memory, and the die had been cast. After a summer as a visiting lecturer at the University of Illinois (where he first held an enzyme—chloroperoxidase—in his hands), Knowles returned to Oxford in the fall of 1962 as Fellow and Tutor of Wadham College (which was a tenured position!). Four years later, he was appointed University Lecturer. At Oxford, Knowles set to work on α-chymotrypsin, and his early studies (*5*) helped to define structural features required for substrate recognition by the enzyme. In a 1965 review of this topic (*6*), he postulated, based on correlations "of binding and inhibition constants with corresponding free energies of transfer from water to non-aqueous solvents," that the "specificity of α-chymotrypsin for different amino acid side-chains [arises] simply from hydrophobic interactions between enzyme and substrate." (Later in the decade, this view was confirmed when the crystal structure of chymotrypsin,with its now familiar specificity pocket, was solved by David Blow's group (*7*).) He then proceeded to analyze the relationship between substrate binding and *catalysis*:

> For specific substrates with *different* amino acid side-chains, it is clear that there is an approximate inverse relationship between $[k_{cat}]$ and $[K_m]$ ... and this leads directly to the hypothesis of "better binding: better reaction" .... That is, just as $[K_m]$ is low for non-polar substrates because of a strong interaction with the non-polar site of the enzyme, so $[k_{cat}]$ is raised, by virtue of the same type of interaction in the transition state of the rate-determining catalytic step ....

(For readers now feeling a bit discomfited, rest assured that earlier in the article, the distinction between a substrate's Michaelis constant and its dissociation constant had been thoroughly discussed.) The essential point quickly followed: "That is, changes in [binding of substrate] are accompanied by greater changes in [the binding of the transition state]." The group continued to publish on chymotrypsin until

1968, focusing primarily on the stereochemistry of the active site. That year, for example, Ingles and Knowles (*8*) posited

> ... that the orienting interaction of the acylamino group of a chymotrypsin substrate is provided mainly by a hydrogen bond between the -NH- group of the acylamino group and an unknown acceptor group in the active site of the enzyme. This interaction orients the susceptible carbonyl group of the acyl-enzyme either favourably (L-series) or unfavourably (D-series) for the subsequent deacylation step, but contributes negligibly to the free energy of binding ....

The group's first paper on pepsin appeared in 1965 when Cornish-Bowden and Knowles (*9*) described a remarkable continuous assay of the enzyme's activity:

> The reaction takes place in a thermostatted bottle, the contents of which are continuously sampled (commonly at 0.235ml./min.) by means of a proportioning pump. The sample stream is mixed with ninhydrin reagent (0.80ml./min.), and bubbles of nitrogen (0.42ml./min.) are introduced into the stream in order to minimize diffusion. The mixture is pumped through a mixing coil and into a heating bath which is maintained at 95°. The mixture takes about 15 min. to pass through the bath, during which time the colour is developed. The mixture is then pumped through a cooling coil and passes via a debubbler into a differential colorimeter, which monitors the absorbance at 570 mμ.

To this day, the mechanism of pepsin remains the subject of debate (*10*), although a consensus on its general features seems finally to have been reached (*11*). In 1970, however, when Knowles published a review on the enzyme (*12*), confusion reigned. He did strive to accommodate the data at hand, however, and proposed a mechanistic scheme

> ... similar to one put forward by Delpierre & Fruton (1965), who suggested 'that a single carboxylate group ($ECOO^-$) of pepsin attacks the carbonyl carbon of the protonated amide ($RCONHR'$) to form reversibly a tetrahedral intermediate ... (which) ... is thought to undergo a reversible four-centre exchange reaction leading to the expulsion of RCOOH and the formation of ECONHR'. The reaction of this product with water (or a carboxylic acid) would liberate NH_2R' (or lead to transpeptidation by 'imino transfer') with the regeneration of $ECOO^-$.'

In 1975, in the group's final paper on pepsin, Newmark and Knowles (*13*) proposed a revised mechanism: "acyl transfer may—for appropriate substrates—be just as important in pepsin catalysis as amino transfer and ... each may occur concurrently."

Knowles can be pardoned for endorsing mechanisms so inelegant (for the one time in his career!) as critical data—not, of course, obtained at Oxford—were later shown to be incorrect. Moreover, during this period, Kitson and Knowles (*14*) had observed that

> ... mechanisms avoiding the unnecessary postulate of acyl-enzyme formation can reasonably be preferred.
>
> The question of direct proof for a covalent *amino*-enzyme intermediate continues to concern us.

Indeed, pepsin is now thought to proceed via a general acid, general base mechanism, involving neither an acyl- nor an amino-enzyme intermediate (*15*). As is characteristic of Knowles, however, he did provide an illuminating commentary on the mechanism he offered in 1970, querying "one may justifiably ask what is the kinetic advantage of breaking the substrate peptide link, only to form another peptide link [the amino-enzyme intermediate] with the enzyme?" Citing Bender's work on the

hydrolysis of phthalamic acid, Knowles concluded that the enzyme takes advantage of the second, protonated, active-site carboxyl group, which is poised to catalyze the now intramolecular hydrolysis of the amino-enzyme intermediate. (Given the intricacy of the proposed pepsin mechanisms, it is no wonder that during this period the group was turning to the calm of triosephosphate isomerase: a simpler system was needed to ask more complicated questions.)

Knowles' publications on chymotrypsin and pepsin reveal a honed perception of catalytic strategies. His rationale for his pepsin mechanism followed upon Westheimer's suggestion (*16*) that enzymes can act as "entropy traps" and his analysis of the relationship between substrate binding and catalysis in chymotrypsin was an early treatment of an idea that reached its apotheosis, a decade later, in Jencks' formulation (*17*) of "intrinsic binding energy." Knowles' insight is evident too in the first of his many review articles on enzymatic catalysis, "The Foundations of Enzyme Action" (*18*), which he coauthored with Freddie Gutfreund while at the Max Planck Institute during the summer of 1966. In addition to providing the initial appearance of the oft-quoted aphorism "[a picture of a horse] does not necessarily tell us how fast it can run," this article is notable for its scholarly and global summary of the "features of enzyme catalysis which are now evident." (Quoting this misusage of the word "which" is intended to show that we were all young once, even Jeremy.) The review concluded:

> In addition to a knowledge of the three-dimensional structure of enzymes, what is required for a full interpretation of their chemical action? The specificity of the chemical configuration involved in the binding of substrates, and the nature as well as kinetic contribution of the subsequent conformational changes, are likely to be elucidated by the methods at present being applied to these problems. Understanding of the chemical mechanisms of the various catalytic processes will eventually depend on the progress of physical organic chemistry in the study of model systems. With a complete knowledge of the structure and dynamic behavior of protein molecules during catalysis, workers will be in a position to design and study much more realistic models of enzyme reactions than has been possible up to now.

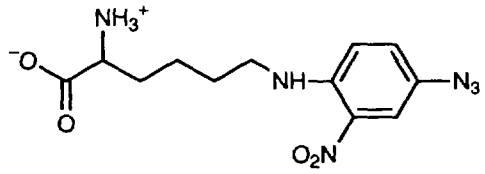
Although the tools available and approaches employed have advanced considerably since the above was written, enzymes continue to occupy a lofty perch, as Knowles suggested in 1991 (*19*):

> And if our bioorganic effort and chemical mimicry continue to fall short of the catalytic potency of today's enzymes, at least we know that nature has been adjusting and refining for rather longer than we have.

By the early seventies, the work on pepsin was drawing to a close, and two new targets, quite different from each other, had become the focus of the group: photoaffinity labeling and triosephosphate isomerase.

PHOTOAFFINITY LABELING

In collaboration with George Fleet and Rodney Porter, the first photoaffinity results were reported in 1969 (*20*). (Here, Westheimer's influence is again clear (*21*).) Rabbits were immunized with a 2-nitro-4-azidophenyl (NAP) derivative, the aryl azide functionality of which is "indefinitely stable" under *in vivo* conditions. The antibodies obtained were purified and treated with radiolabeled NAP-lysine, and the complex was photolyzed.



NAP-lysine

As Knowles described in a 1972 *Accounts* review (*22*):

> Most of the label was found in the heavy chains, and after peptide digestion and purification two radioactive peptides, X-Ala-Arg and Phe-Cys-Y-Arg, were isolated. X and Y are modified amino acids of presently unknown structure.
>
> Information of this kind is not very helpful by itself, but a study of the sequence of the heavy chain of pooled normal IgG by Porter and his group revealed in positions 91–94 the sequence: -Phe-Cys-Ala-Arg-. It therefore appears probable that X is a modified cysteine residue and that Y is a modified alanine residue. However, more significant than the apparent success of this indiscriminate reagent in identifying a part of the antibody combining site, residues 91–94 are adjacent to the major "hypervariable" region of the heavy chain. That is, we have strong support for the premise that one of the hypervariable regions in the IgG molecule is at least partly responsible at the molecular level for antibody specificity.

At this time, which was prior to the advent of both monoclonal antibodies and gene sequencing, the above conclusion was extraordinarily significant. Ironically, since a polyclonal preparation was (perforce) utilized, the experiment would likely have failed to yield a tractable peptide fragment if labeling had occurred within the hypervariable region itself, where sequence heterogeneity can be extensive. Additional work on this system was reported in 1974 by Smith and Knowles (*23*), who studied the labeling of the anti-NAP antibody with both NAP-lysine and the closely related 5-azido isomer. The results obtained demonstrated that

> ... the discrimination of the labelling is very high, since generation of nitrene at centers only about 0.2 nm apart ... leads to a mutally exclusive labelling pattern. This stresses the effectiveness of the photolabelling approach and suggests that ... [it] can be used both for the 'mapping' of sites in homogeneous systems and for probing of the basis of heterogeneity in systems that are not.

An enzyme, of course, provides such a homogeneous system, and soon thereafter, Bridges and Knowles (*24*) reported the labeling of the specificity pocket of that familiar friend α-chymotrypsin.

The group continued to exploit photoaffinity reagents throughout the decade, and diazirine derivatives, which Smith and Knowles (*25*) introduced in 1973, provided the basis for many of the later studies. Upon photolysis, diazirines yield carbenes, which are more reactive and less selective than the nitrene functionality generated from the earlier-employed acyl azide derivatives. By 1980, for example, Bayley and Knowles (*26*) had developed the hydrophobic species adamantanediazirine as a reagent that "can be used to label those segments of intrinsic membrane proteins that are in contact with the hydrocarbon core of the lipid membrane." The extraordi-

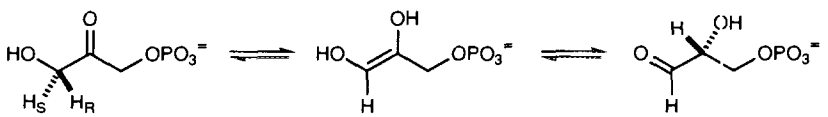
nary care with which data generated in the Knowles group have been analyzed is again evident from the penultimate paragraph of this paper, which begins:

> Adamantanediazirine, though useful, is clearly not the "perfect" hydrophobic reagent. Carbenes exhibit some selectivity toward different functional groups, and we have not yet shown which groups within the bilayer are labeled. Indeed, it is possible that a hydrophobic reagent could label just outside the bilayer .... The selectivity of the carbene presents a further problem .... In short, we cannot assume that all hydrophobic peptides are equally reactive toward the reagent ....

As the Knowles group was exploiting photoaffinity reagents to identify functionally important amino acid residues, it also published a number of papers on the purification and properties of aminoacyl-tRNA synthetases (see, for example, Refs. *27* and *28*). Triosephosphate isomerase, however, soon became the main focus of the lab's work.

TRIOSEPHOSPHATE ISOMERASE

Triosephosphate isomerase catalyzes the now famous interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (GAP) via, as originally suggested by Irwin Rose (*29*), a *cis*-enediol phosphate intermediate:



[1]

dihydroxyacetone phosphate enediol phosphate D-glyceraldehyde 3-phosphate

A single base mediates the proton transfer, and the *pro-R* hydrogen is removed from DHAP.

In a signal moment, the group's first publication on triosephosphate isomerase appeared in 1970 (*30*). This paper described the use of the active-site-directed reagent bromohydroxyacetone phosphate (BHAP). Treatment of the enzyme with ^{32}P-labeled BHAP resulted in less than stoichiometric incorporation of label, whereas labeling with [^{14}C]BHAP resulted in incorporation of the expected two equivalents of label per dimer. Proteolysis of the ^{14}C-labeled rabbit enzyme yielded "a multiplicity of radioactive peptides," but the redoubtable chicken enzyme yielded "a single radioactive peptide fragment." Later that year (*31*), the ^{14}C-labeled peptide was identified as Ala-Tyr-Glu-Pro-Val-Trp, in which the site of attachment "is, most probably, tyrosine." Additional studies (*32*) then confirmed what had earlier been inferred: BHAP initially reacts with the neighboring glutamate residue and subsequently migrates to the tyrosine residue. In this rearrangement, the phosphate group is lost. This glutamate residue, glutamate$_{165}$, was thus assigned as the active-site base. In 1974, Webb and Knowles (*33*) presented evidence that triosephosphate isomerase also effects electrophilic catalysis of the isomerization reaction. Crystallographic data from David Phillips' group (*34*) later allowed identification of the residues lysine$_{13}$ and histidine$_{95}$ as the possible active-site electrophiles. From the

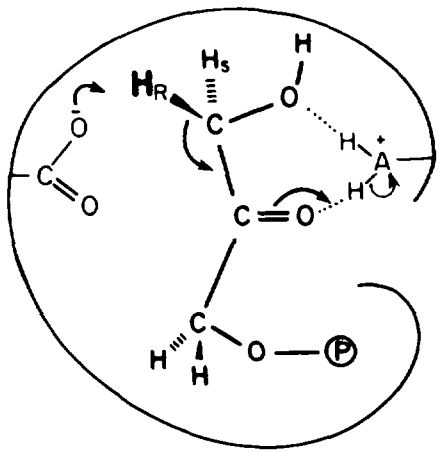


FIG. 1. Putative formulation of the complex of dihydroxyacetone phosphate with triosephosphate isomerase. The carboxylate group of Glu_{165} abstracts the *pro-R* hydrogen, assisted by an electrophilic catalyst as shown. (Reprinted with permission from J. R. Knowles and W. J. Albery (1977) *Acc. Chem. Res.* **4**, 105–111. Copyright 1977 American Chemical Society.)

Fourier transform infrared spectrum of the enzyme–DHAP complex, Belasco and Knowles (*35*) unequivocally established in 1980 the role of carbonyl group polarization in the isomerase-catalyzed reaction. (Belasco and Knowles published a similar FTIR study of the aldolase–substrate complex soon thereafter (*36*).) As shown in Fig. 1 (*37*), the above data allowed a good picture of the enzyme–DHAP complex to be constructed. (This figure, like all in this review, has been reproduced from a Knowles paper, and the original legend has been transcribed.)

Work on the kinetics of the triosephosphate isomerase-catalyzed reaction was also proceeding apace, and a number of publications (see, for example, (*38*)) from the early seventies augured the appearance of the free-energy profile, which was shortly forthcoming. During this period, Knowles spent 1969 at Yale as a visiting professor and 1973 at Harvard as the Sloan Visiting Professor. In 1974, he permanently joined the Harvard faculty as Professor of Chemistry, and 5 years later he was named Amory Houghton Professor of Chemistry and Biochemistry. In an interview conducted just before his arrival at Harvard (*39*), Knowles noted:

> Why am I going? Having been in Oxford for so long, I was very happily settled, both departmentally and socially, and Oxford is a splendid place to live. But the challenge of a new and exciting environment in the end became dominant.

He also took this opportunity to comment upon his earlier observation regarding horses and pictures and speed:

> A molecular structure is an absolute *sine qua non* for any complete mechanistic description of enzyme catalysis .... But I do not believe that structure alone is enough and I have been teased considerably for a remark made in about 1966 ....

Indeed, crystallographic data were to play a crucial, but not preeminent, role in the triosephosphate isomerase work that would appear in the 2 decades to follow.

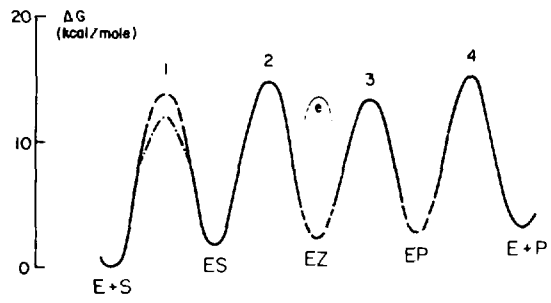


FIG. 2. The free-energy profile for the reaction catalyzed by triosephosphate isomerase. The standard state is 40 μM (which is the known concentration of triosephosphates *in vivo*). Broken lines indicate the limits on those species that may be kinetically insignificant. The line ---- for transition state 1 is the diffusion-controlled limit (assuming $k_1 = k_{-4}$). The barrier labeled "e" represents that for the exchange of the proton from the conjugate acid of the enzyme's catalytic base (in the enzyme–enediol intermediate) with a proton from the medium. (Reprinted with permission from W. J. Albery and J. R. Knowles (1976) *Biochemistry* **15,** 5627–5631. Copyright 1976 American Chemical Society.)

In 1976, eight successive papers in *Biochemistry* (*40–47*) described the elucidation and the analysis of the free-energy profile of the isomerase-catalyzed reaction. The essential contribution of John Albery to this work was revealed almost immediately, for example, in this paragraph from the first of the papers:

> A particular problem arises with the isotopic exchange reaction described by k_5 (Scheme I). Like all other k_n, k_5 describes the all-H system:
>
> $$EZ(H) + H_2O \underset{k_5}{\overset{k_5}{\rightleftharpoons}} EZ(H) + H_2O$$
>
> Experiments in which deuterium is picked up from the solvent require such high atom fractions of D that the interpretation of the results is complicated by solvent isotope effects. We therefore will not consider experiments performed in D_2O or in mixtures of H_2O and D_2O. So the step, $EZ \rightarrow EZ'$, is only important when T is being picked up from the solvent:
>
> $$EZ(H) + HTO \xrightarrow{\Phi_5 k_5} EZ'(T) + H_2O$$
>
> The rate constant for this process is $k_5\Phi_5$ since EZ is not isotopically substituted and the T is coming from a solvent site, where $\Phi = 1$ by definition, to the transition state (Φ_5). Note that when EZ and EZ′ are in equilibrium:
>
> $$k_5\Phi_5 \mathbf{x} ez = k_5(\Phi_5/\Phi_{EZ})(1 - \mathbf{x})\, ez'$$
>
> where **x** is the atom fraction of T in the solvent. Hence
>
> $$\Phi_{EZ} = \frac{ez'(1 - \mathbf{x})}{ez\mathbf{x}}$$
>
> which agrees with the eq 1.1 given above.

(Albery's contribution is also apparent, for example, from even a cursory glance at the fifth figure in this first paper!) In the seventh of the papers, the free-energy profile of triosephosphate isomerase was revealed (Fig. 2, where S is DHAP, P is GAP, and Z is the enediol intermediate).

Two features of the triosephosphate isomerase-catalyzed reaction made it especially amenable to a detailed kinetic study. First, as Rose's group had demonstrated (*29*), the mechanism involves an intermediate that exchanges protons with solvent water, and second, as Albery and Knowles explained in a 1977 *Accounts* article (*37*):

> The catalyzed reaction is chemically simple .... The overall equilibrium constant is near enough to 1 to allow the catalyzed reaction to be studied in either direction ... this possibility provides new information since effective rate- and product-determining steps are different for the forward and reverse reactions.

Although an enormous number of kinetic measurements were required to elucidate the profile, the titles of the second through sixth papers suggest the overall strategy: "Energetics of Triosephosphate Isomerase: The Fate of the 1(*R*)-^{3}H Label of Tritiated Dihydroxyacetone Phosphate in the Isomerase Reaction," "Energetics of Triosephosphate Isomerase: The Appearance of Solvent Tritium in Substrate Dihydroxyacetone Phosphate and in Product," "Energetics of Triosephosphate Isomerase: The Appearance of Solvent Tritium in Substrate Glyceraldehyde 3-Phosphate and in Product," "Energetics of Triosephosphate Isomerase: Deuterium Isotope Effects in the Enzyme–Catalyzed Reaction," and "Energetics of Triosephosphate Isomerase: The Nature of the Proton Transfer between the Catalytic Base and Solvent Water." As summarized by Albery and Knowles (*37*):

> In all, four kinds of experimental situation have been studied: (1) Specifically tritiated substrate in ^1H_2O, the specific radioactivity of product (transfer) and of remaining substrate (enrichment or depletion) being measured as a function of the extent of reaction; (2) unlabeled substrate in tritiated water, the specific radioactivity of remaining substrate (exchange) and of product (discrimination) being measured as a function of the extent of reaction; (3) measurement of the initial steady-state rates for specifically deuterated substrates in ^1H_2O; and (4) measurements of the steady-state rates for unlabeled substrates in ^1H_2O.

However, in spite of this tremendous effort, the free-energy profile was not quite complete. As Raines and Knowles (*48*) noted in 1987, the derivation had assumed the existence of just one form of the enzyme. Yet,

> Catalysis is a cyclic process. After facilitating the conversion of substrate to product, the catalyst must return to the form that accepts substrate so that the next cycle can begin. The regeneration of the substrate-accepting form of a catalyst may not be a trivial process ....

The tracer perturbation method of Hubert Britton (*49*) was thus utilized to determine whether the interconversion of two unliganded forms of triosephosphate isomerase—one that binds DHAP, the other GAP—is kinetically significant. Indeed, at high substrate concentration it is, having a rate constant of approximately 10^6 s^{-1}.

In the final of the eight *Biochemistry* papers, Albery and Knowles addressed the issue of catalytic "perfection" by defining an efficiency function, which has a value of 1.0 for a perfect catalyst and becomes smaller as catalytic efficiency decreases. (The following year, Albery and Knowles (*50*) again addressed the issue of perfection in a review article.) The value of the efficiency function for triosephosphate isomerase is 0.6 while that for the simple carboxylate catalyst acetate ion (which may be viewed as a truncated form of the enzyme) is 2.5×10^{-11}; hence, the catalytic effectiveness of triosephosphate isomerase is over 10^{10}-fold greater than that of

acetate. (In 1984, Richard (*51*) showed that the rate constants utilized by Albery and Knowles for the acetate ion-catalyzed reaction were incorrect; nonetheless, they were *almost* correct, and the original calculations will be presented here.) Albery and Knowles postulated that this enormous increase in catalytic effectiveness evolved by the blending of three processes. In the first and least difficult to achieve, termed "uniform binding," the enzyme binds the ground states and transition states of the acetate ion-catalyzed pathway equally well (in essence acting as an entropy trap). When uniform binding is optimized, the value of the efficiency function rises to 3×10^{-6}. In the second process, termed "differential binding," the enzyme discriminates between bound intermediates so as to equalize the rate of turnover of each intermediate. After differential binding is optimized and uniform binding is readjusted, the value of the efficiency function increases to 1.5×10^{-4}. In the third and most difficult process, the "catalysis of an elementary step," the free energies of the kinetically significant transition states are lowered with respect to those of the kinetically significant intermediates. Ultimately, after uniform and differential binding are further readjusted, the free energy of all the enzyme-bound transition states becomes lower than that of the higher of the two diffusive transition states, and the free energy of all the enzyme-bound ground states becomes higher than that of the more stable unbound species, which in this case is DHAP. These two conditions are met for the interconversion of DHAP and GAP as catalyzed by triosephosphate isomerase, and the value of the efficiency function for present-day triosephosphate isomerase is 0.6. Although a value of 1.0 is deemed "perfect," little advantage is gained by further accelerating the chemical steps once a diffusive barrier has become rate limiting. As Albery and Knowles stated (*37*):

> Once the enzyme has (a) lowered the free energies of all transition states below that of [a diffusive] step ... and (b) raised the free energies of all intermediate states above that for the (enzyme + [DHAP]) state, then there is nothing more it can do.

(Jeremy's later reference (*52*) to *Isaiah* 40:4 as a commentary on catalytic perfection is typical.)

In 1989, following the lead of Jik Chin (*53*), Burbaum *et al.* (*54*) generalized the derivation to include enzymes that have "become optimized for catalysis under *any* given steady-state concentrations of substrate and product." The original efficiency function had treated only enzymes, such as triosephosphate isomerase (and many others), that operate at equilibrium *in vivo*. A much-discussed result of this original derivation was that the equilibrium constant for productively bound substrates and products, K_{int}, will be unity for a perfectly evolved enzyme. As noted by Chin, however, this prediction does not hold for enzymes operating away from equilibrium. The theory presented in 1989, however, does allow the optimum K_{int} to be calculated under all possible conditions:

> Two classes [of enzyme] are apparent. For an enzyme that operates near equilibrium, the catalytic efficiency is sensitive to the value of K_{int}, and the optimum value of K_{int} is near unity. For an enzyme that operates far from equilibrium, the catalytic efficiency is less sensitive to the value of K_{int}, and K_{int} assumes a value that ensures that the rate of the chemical transformation is equal to the rate of product release. In each of these cases, the internal thermodynamics is "dynamically matched", where the concentrations of substrate- and product-containing complexes are equal at the steady state in vivo.

Burbaum and Knowles (*55*) then analyzed the internal equilibrium constants of nearly 20 enzymes and concluded that "these data are consistent with the theory for kinetically optimized enzymes ...."

Also in 1976, Knowles took stock of his trade in an essay entitled "Whither Enzyme Mechanisms?" (*56*). He began:

> The ubiquity of papers in the biochemical literature that claim to present the 'mechanism of action' of particular enzymes might suggest to the uninitiated that we understand how these catalysts accelerate the rates of reactions that they mediate. We do not.

Given that the free-energy profile for triosephosphate isomerase was to appear just months later, this statement may seem odd. Knowles continued, however:

> ... but understanding includes the capacity to predict, and we should not be satisfied with our understanding unless it provides the basis for prediction about the pathways and rates of enzyme-catalyzed reactions.

Daunting words, to be sure, even when viewed from today's vantage point.

To probe the different kinds of binding discrimination proposed by Albery and Knowles in their analysis of catalytic perfection, the group turned to the tools of protein engineering, which by the mid eighties had firmly entered the enzymologist's ken. In collaboration with Walter Gilbert's group, the glutamate$_{165}$-to-aspartate (E165D) mutant—in which the active-site base is retracted by a bit less than 1 Å—was generated (*57, 58*), and its free-energy profile was determined (*59*). (Only 8 of the 16 possible kinetic experiments originally described by Albery and Knowles (*39*) were performed, however, with the mutant isomerase!) Although the mutant is approximately 1000 times less active than the wild-type enzyme (that is, it is "sluggish"), its catalytic mechanism is analogous. Its free-energy profile was analyzed thus:

> Our mutation has made the enzyme less perfect, taking, in a sense, an evolutionary step backward. The two enolization steps in the reaction have each been slowed by more than 3 orders of magnitude, and the overall reaction is now limited by a chemical transition state that involves covalency changes rather than one that involves substrate diffusion. Interestingly, the free energies of the bound intermediates ... have changed little with respect to the wild-type enzyme.

That is, alteration of glutamate$_{165}$ revealed that this residue functions primarily in the "catalysis of an elementary step." (The E165D mutant later served as an important control in experiments using viscosogenic agents that proved that the wild-type enzyme is diffusion-limited (*60*).) The group quickly discovered, however, that not all triosephosphate isomerase mutants are as well behaved. For example, in collaboration with Gregory Petsko's group (*61*), the reaction catalyzed by the histidine$_{95}$-to-glutamine mutant was shown to proceed via an altered mechanism in which "there is a change in the pathways of proton transfer." (The activity of this mutant is approximately 400-fold lower than that of the wild-type.) Although these latter experiments employed a mutant form of the enzyme from yeast, rather than chicken muscle, Nickbarg and Knowles (*62*) had demonstrated that the "resemblance between the free energy profile of the [two isomerases] is close, in spite of the considerable difference that separates yeasts from vertebrates on the phylogenetic tree."

The creation of mutant, sluggish triosephosphate isomerases allowed, of course, for the possibility of generating second-site pseudo-revertants, and, as Hermes *et al.* (*63*) noted in 1987, for the study of "the *forward* evolution of enzyme function." (In the group's futile search for a preexising "less than perfect isomerase," triosephosphate isomerases from calf, chicken, human erythrocytes, yeast, *Baccilus stearothermophilis*, coelacanth, *Ankistodemus*, and *Methanobacterium thermoautotrophicum* were all shown to be equally active! For reasons now obscure, the ginko tree isomerase was not studied.) Upon chemical mutagenesis and selection, Hermes and Knowles (*64*) isolated a second-site suppressor mutant of E165D in which $serine_{96}$ is replaced by proline; this enzyme is 23 times more active than the initial mutant, but still 19 times less active than wild-type. (Remarkably, the same second-site change also increases the activity of the $histidine_{95}$-to-asparagine mutant.) The above data were analyzed as follows:

> ... the two free energy barriers that relate to the optimization of uniform binding are, for the wild-type enzyme, approximately equal. In the mutant having aspartic acid in place of glutamic acid-165, these two free energy differences are *unequal*, and this mutant ... could most readily improve as a catalyst by the tighter (uniform) binding of all the internal states. In essence, this seems to have occurred: the pseudo-revertant enzyme is indeed closer to meeting the uniform binding condition.
>
> While it would clearly be improper to suggest that this single result indicates the validity of our earlier theories, it is gratifying that in this first case, we have found the type of change in the reaction energetics that we had suggested would be the most facile.

(Knowles' 1987 review article on site-directed mutagenesis (*65*) nicely summarizes the role that particular amino acid residues can play in the three types of binding interactions.) A more detailed analysis of suppressor sites of the E165D mutant, in which its gene was "subjected to random mutagenesis over its whole length by using 'spiked' oligonucleotide primers," was published in 1990 (*66*). Six second-site mutants—G10S, S96P, S96T, E97D, V167D, and G233R—which ranged in catalytic improvement from 1.3- to 19-fold were isolated. That six pseudo-revertants were generated using the spiked oligonucleotide method contrasts to the one produced chemically (S96P—described above), and implies that the "spiked primer method is clearly superior in the exploration of sequence space." As shown in Fig. 3 (*67*), all six mutations are in or near the active site, suggesting that

> ... it may turn out for enzymes such as triose-phosphate isomerase, where there is no evidence for information transfer either within or between protein subunits, that only local changes in the first or second shell of amino acid residues near the active site will affect the specific catalytic activity... Further, the idea of a range of pendant catalytic groups from a stable scaffolding is made attractive by the growing list of enzymes... that catalyze a variety of different chemical reactions but that all have their active sites at the C-terminal end of an eightfold β-barrel, the archetype of which is triose-phosphate isomerase.

The following year, Blacklow *et al.* (*67*) generated all 14 possible "triple mutants," each having the intial E165D mutation and a pair of the second-site mutations above. In 6 of these triple mutants, the kinetic effects of the two suppressor mutations were additive; in the other 8, less than the combined effect was seen. In no case did two paired mutations act synergistically.

While these studies on mutant isomerases were progressing, the group was also

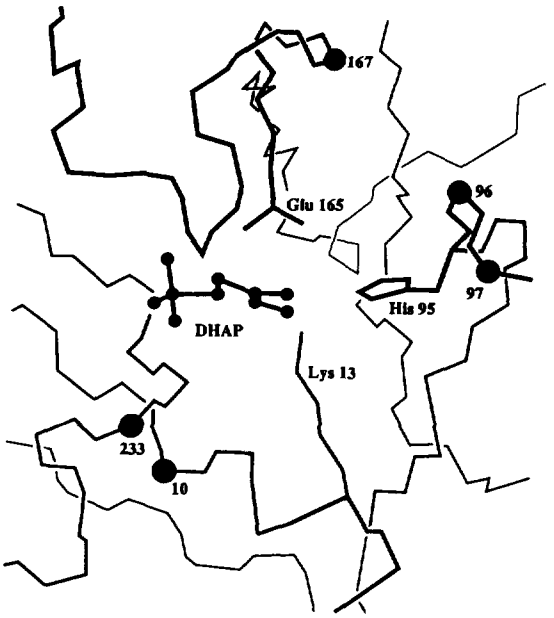


FIG. 3. Active site of wild-type triosephosphate isomerase. The coordinates are those of the native yeast enzyme with phosphoglycolohydroxamate bound. The position of the inhibitor relative to the enzyme is consistent with abstraction of the 1-*pro*-(*R*) proton of the substrate by glutamate-165, and with an electrophilic contribution to catalysis by histidine-95 and lysine-13. The Cα carbons of residues where second-site suppressor mutations occur are highlighted on the protein backbone. (Reprinted with permission from S. C. Blacklow, K. D. Liu, and J. R. Knowles (1991) *Biochemistry* **30,** 8470–8476. Copyright 1991 American Chemical Society.)

uncovering new information about the functioning of the native enzyme. In 1991, Knowles published two reviews on triosephosphate isomerase, "Enzyme Catalysis: Not Different, Just Better" (*19*) and "To Build a Better Enzyme ..." (*68*). In both, he focused on four features of the enzyme's catalytic "apparatus": the active-site "loop," glutamate$_{165}$, histidine$_{95}$, and two conserved α-helices. With the exception of glutamate$_{165}$, these topics provided the impetus for papers on triosephosphate isomerase published by the Knowles group during the current decade (see, for example, Refs. *69–71*). How then does triosephosphate isomerase effect catalysis? Let me quote extensively from the first of the reviews, beginning with "Intermediate sequestration":

> ... a loop of ten or eleven amino acids between residues 166 and 176 moves about 7 Å from an 'open' position in the unliganded enzyme into a 'closed' position when there is substrate or inhibitor bound at the active site .... It thus appears that the loop closure preferentially stabilizes the enediol phosphate intermediate, as well as the two transition states that flank it. In the absence of a proper lid, the enediol phosphate intermediate is lost from the truncated enzyme into solution, where it rapidly decomposes ....

In the section "A poised base," Knowles noted:

> ... it is clear that the oxygens of the carboxylate of Glu 165 are beautifully positioned for the

> proton abstractions required .... This arrangement is exquisite for four reasons. First, the fit is snug .... Second, the carboxylate group is bidentate, which means the proton can be removed either from C-1 of dihydroxyacetone phosphate ... or from C-2 of glyceraldehyde 3-phosphate ... with minimal motion of the catalytic base. Third, the stereoelectronic requirement for rapid enolization ... is nicely satisfied by the active site arrangement of triosephosphate isomerase. Fourth, the argument of Gradour that a *syn* orbital of a carboxylate ion is much more basic than an *anti* orbital ... has also been recognized in the [active-site] configuration ....

Let us, for the moment, skip over the third section, "An unexpected electrophile," to the fourth, "Well-aimed helices":

> Triosephosphate isomerase has two conserved α helices that are directed towards the active site. One helix is trained on His 95, the imidazole ring of which is at the positive end .... The aim is precise ... and the arrangement of this stretch of helix accounts for the perturbation [of the His 95 pK_a] very well.
>
> The second helix that points into the active site has its positive end aimed at the phosphate group of the substrates .... This arrangement too, is attractive, because there is only one positively charged group at the active site (Lys 13), and yet the substrates bind as their phosphate di-anions.

(Based upon crystallographic data, Lolis and Petsko (*72*) have demonstrated that hydrogen bonds to at least four main-chain amides also serve to stabilize the bound phosphate group.) We now return to the third section:

> [On the basis of the above discussion], the reader would be forgiven for concluding that [the enzyme employs] a precisely positioned general base (the carboxylate of Glu 165) and a well-placed general acid (the imidazolium of His 95), which are poised for 'push–pull' catalysis. Yet this is not how things are .... The crystal structure of the isomerase indicates from the pattern of hydrogen bonds at the active site that the imidazole ring of His 95 is not protonated at neutral pH; this implies that the enzyme uses imidazole rather than the much more electrophilic imidazolium as its acid component .... Using ^{15}N NMR, we found that the pK_a of His 95 is lowered from 6.7 in the denatured protein to less than 4.5 in the native enzyme, and we observed the formation of a new hydrogen bond (presumably to substrate) from the neutral imidazole ring ... indicating that the enzyme does use the less acidic neutral imidazole as its electrophile. Why, when everything else about this enzyme seems chemically so reasonable, it should fail to use the more powerful electrophile, remains a puzzle.

But it didn't remain so for long! Taking a lead from Gerlt and Gassman (*73*), Zhang *et al.* (*74*) concluded in 1994:

> A neutral imidazole should have a pK_a around 16 ... (the helix dipole may depress this value somewhat), which is close to that expected for the enediol. Matching the pK_a's of the proton donor and acceptor would lead to rapid proton transfer followed by formation of a short, strong hydrogen bond between His 95 and the intermediate, stabilizing this species ....

Such "low-barrier hydrogen bonds" have recently been reviewed by Cleland and Kreevoy (*75*), who similarly analyzed their significance with regard to triosephosphate isomerase:

> The group hydrogen bonding to the carbonyl oxygen of the substrate is a neutral histidine. This discovery was a surprise, but the reason for this arrangement becomes obvious if one considers the necessity to match the pK_a's of the enediol and the histidine to form a low-barrier hydrogen bond to the intermediate. The pK_a of the enediol will be ~14, which matches the pK_a of neutral imidazole but not the pK_a of protonated imidazole (~6) ...

In sum, our current understanding of the catalytic functioning of triosephosphate

isomerase is comprehensive, and it may be nearly complete. Even the role of neglected $lysine_{13}$ has recently been elucidated (*76*). Some mysteries, however, apparently still remain: for example, the importance of low-barrier hydrogen bonds has recently been severely challenged (*77, 78*). Nonetheless, the Knowles group, in its more than 2 decades of work, has provided a compelling picture of the catalytic strategies employed by triosephosphate isomerase, a picture that will, I believe, require few additional brush strokes.

By now the reader may have concluded that once the study of triosephosphate isomerase had commenced, little else occupied the attention of the Knowles lab. Remarkable, therefore, is the fact that from 1978 to 1985 the group published only two papers on the enzyme (*35, 79*). Instead, during this period, research focused in three other arenas: the energetics of proline racemase, the inhibition of β-lactamase, and the mechanism of phosphoryl-group transfer. Also during this period, a series of kinetic and mechanistic studies, all familiar to one schooled on triosephosphate isomerase, were undertaken on ribulose bisphosphate carboxylase (*80–82*). The data obtained, while not allowing for the complete delineation of the latter enzyme's free-energy profile, confirmed "the existence of the enediol intermediate and set limits on the range of permissible free-energy levels for the transition states in the catalyzed reaction."

PROLINE RACEMASE

In 1986, after almost a decade of effort, the free-energy profile for proline racemase appeared in the last of, this time, just seven consecutive *Biochemistry* papers (*83–89*). Critical earlier studies on proline racemase by the Abeles group (*90*) had suggested that the enzyme, which does not utilize pyridoxal phosphate or an N-terminal pyruvoyl residue, follows the pathway shown in Fig. 4 (*83*). The racemiza-

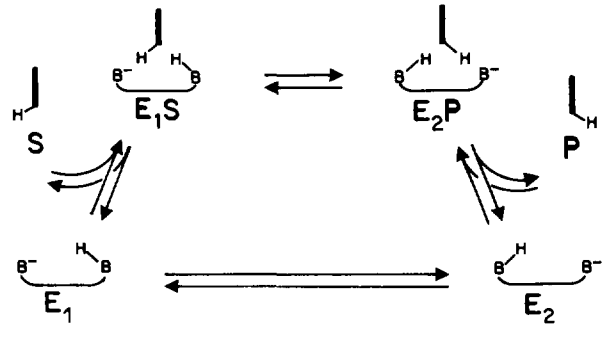


FIG. 4. Proline racemase reaction. The two enzyme bases (B) catalyze the interconversion of the two enantiomers of proline (the proline ring is shown edge-on as a heavy bar). (Reprinted with permission from L. M. Fisher, W. J. Albery, and J. R. Knowles (1986) *Biochemistry* **25,** 2529–2537. Copyright 1986 American Chemical Society.)

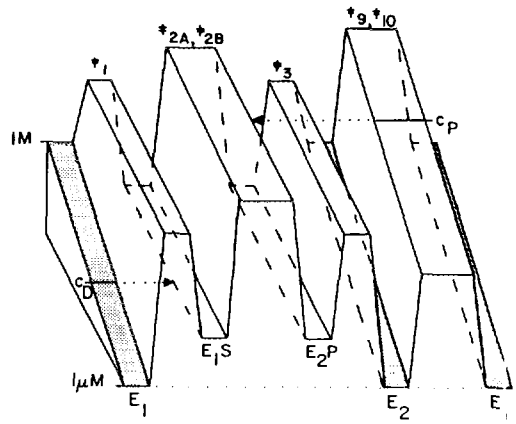


FIG. 5. Free energy profile for proline racemase as a function of substrate concentration. At the front of the diagram ([S] = [P] = 1 μM) the system is saturated, and the rate-limit ng free-energy difference is between E_1 and ‡2. At the back of the diagram ([S] = [P] = 1 M) the system is oversaturated, and the rate-limiting free-energy difference is between E_1S or E_2P and ‡9 or ‡10. Between the dip-switch and peak-switch concentrations (c_D and c_P) the system is bound state saturated with the rate-limiting free-energy difference between E_1S and ‡2. (Reprinted with permission from W. J. Albery and J. R. Knowles (1986) *Biochemistry* **25,** 2572–2577. Copyright 1986 American Chemical Society.)

tion proceeds via a "two-base" mechanism, in which two forms of the enzyme, presumably differing in their protonation states, recognize the two enantiomers. Notably, the k_{cat}/K_m for proline racemase is 9×10^5 M^{-1} s^{-1}, far below the diffusion limit, and hence "perfection" was a moot point—throughout the seven papers, the word does not, I believe, appear even once. The enzyme's interesting features were to be found elsewhere. With the mechanistic scheme in place and with the theoretical insights of John Albery at hand, the work could commence. Although the resulting profile, which is shown in Fig. 5 (*89*), is similar in appearance to that of triosephosphate isomerase (especially if, for the moment, one ignores the extra dimension!), its derivation was quite different, primarily for three reasons. First, as is implicit in the mechanistic scheme shown in Fig. 4, release of the substrate-derived proton is slow relative to release of proline product, thus precluding the use of "exchange–conversion" experiments. Second, the existence of two kinetically significant forms of the free enzyme, each of which appears explicitly in the profile, was a starting point in the proline racemase analysis, rather than the *coup de grâce*, as with triosephosphate isomerase. As noted in the first paper in the series:

> ... we consider an enzyme-catalyzed reaction involving one substrate S, one product P, and two forms of the enzyme E_1 and E_2 .... In any reaction involving enzyme-catalyzed conversion of S to P, it is unlikely that the enzyme will remain unchanged.

And third, all of the kinetics were run under reversible, rather than irreversible, conditions. Again, from the first paper:

> The experiments are performed under reversible conditions where the approach to equilibrium

> is monitored continuously [using a polarimeter]. Since after a short time the product is also present, it is the *net* flux that is measured when the reaction is followed under reversible conditions.

The importance of studying enzymes operating under reversible conditions was masterfully explained, in writing best left unalloyed, in the last of the seven papers:

> In general, we know that saturated behavior for an enzyme (where the reaction rate is independent of substrate concentration) may arise when the slowest step is the conversion of an enzyme–substrate complex to a transition state also involving liganded enzyme. This is the classical explanation of the saturation phenomenon, which we may call bound state saturated. Alternatively, however, precisely the same steady-state behavior will be seen for the entirely different situation in which the rate of reaction becomes independent of substrate concentration because the rate-limiting transition state is that for the interconversion of the two forms of free enzyme. This is called free state saturated ....
>
> [Hence, when] the interconversion of free enzyme forms is rate limiting, one would never discover this from steady-state kinetics under irreversible conditions.... To limit kinetic investigation, therefore, to reactions under irreversible conditions can be seriously misleading, in the sense that the V_{max} may measure a process that has nothing to do with reaction of substrate to product. If, in contrast, reactions are studied under reversible conditions where the substrate and product are present at concentrations near their equilibrium values, we may not only evaluate the nature of V_{max} at saturation, but also encounter a new regime, *oversaturation*, in which the reaction rate is limited by the reaction of E_1S and E_2P over the transition state between E_2 and E_1. It should, moreover, be emphasized that the majority of enzymes operate in vivo under reversible conditions: only those enzymes that are control points in metabolic pathways experience substrate and product levels that are very different from their equilibrium values ....

Thus the need for the third dimension in Fig. 5 becomes clear: it marks the change in the free-energy profile as a function of substrate and product concentration, which are identical in this instance. That the transition state between E_1S and E_2P is labeled twice (as $^{\ddagger}2A$, $^{\ddagger}2B$) denotes the fact that the proton transfers required for the interconversion of D- and L-proline are probably not synchronous; rather, the reaction is thought to proceed via the proline carbanion (*89*). Similarly, the interconversion of E2 and E1 (transition state $^{\ddagger}9$, $^{\ddagger}10$) also likely involves more than one elementary step. The "dip-switch" and "peak-switch" concentrations, which are indicated in Fig. 5 as c_D and c_P, respectively, denote two important thresholds (*89*):

> The profile shows the "dip-switch concentration"... where the free energies of the free enzyme forms E_1 and E_2 match those of the liganded states E_1S and E_2P. At yet higher substrate levels, we come to the "peak-switch concentration" where the free energy of the highest transition states of steps 1–3 ... matches that of the highest transition state of steps 9 and 10 .... It is these defined concentrations that divide the behavior of the enzyme into the three regimes: unsaturated, saturated, and oversaturated.

(Many readers may be unaware that "dip-switch" is the British term for the headlight-dimmer control on an automotive dashboard, a linguistic crime that Albery and Knowles were clearly willing to abet.)

The catalytic bases that mediate the proton transfers were ingeniously identified as cysteine residues on the basis of their ground-state fractionation factors, which were determined to have a value of approximately 0.55. As Belasco *et al.* (*87*) stated:

> That is, the *enzyme-bound* protons in the enzyme–substrate and enzyme product complexes

> have unusually low fractionation factors ... the only stable protonic sites having fractionation factors much less than 1 are thiols, which have values near 0.5 .... On this basis, therefore, *we conclude that both E_1S and E_2P have one of the two protons on a thiol group, the other being the carbon-bound proton of the substrate.* That is, the two catalytic bases of proline racemase are thiols. This finding is entirely consistent with the suggestion, on the basis of chemical modification studies, that the two active groups of proline racemase are cysteine thiols, one contributed by each of the two protein subunits .... While such modification experiments can only prove the existence of particular groups at the active site as distinct from their functional importance in catalysis, our present experiments indicate that these thiols are indeed responsible for proton abstraction and delivery in the enzyme-catalyzed reaction.

That the catalytic bases are cysteines was further established by the absence of a detectable *solvent* isotope effect in the proline racemase-catalyzed reaction.

In addition to providing detailed mechanistic information on the enzyme, the group's work on proline racemase stands as the first complete kinetic treatment of an enzyme-catalyzed reaction under reversible conditions. And, 1 year after the appearance of the above work, Albery and Knowles (*91, 92*) presented a theoretical analysis for *any* reversible enzymatic reaction, including those for which the equilibrium constant is not unity.

The energetic and mechanistic issues raised by proline racemase are numerous, and they proved too compelling not to revisit. In 1993, Gallo and Knowles (*93*) reported the purification and cloning into *Escherichia coli* of glutamate racemase from *Lactobacillus fermenti.* Kinetic and site-directed mutagenesis studies (*94, 95*) confirmed that this racemase also functions using a two-base mechanism, again involving cysteine residues. An oversaturated regime could not be detected, however, even with substrate concentrations as high as 100 mM, a result suggesting that the "two enzyme forms are rapidly interconverting under physiological conditions."

β-LACTAMASE

The group's work on β-lactamase began where much enzymatic work now concludes: with the study of a mutant enzyme. In 1976, Hall and Knowles (*96*) reported the isolation, after chemical mutagenesis and selection, of an *E. coli* RTEM strain that expressed a β-lactamase with altered substrate specificity: activity against cephalosporin derivatives was enhanced, while that against penicillin derivatives was diminished. Although the mutant enzyme contains just a single amino acid substitution—alanine$_{237}$ to threonine (*97*)—a molecular interpretation for the altered activity was precluded by the unavailability of a high-resolution crystal structure. (The 1.8-Å structure of the RTEM-1 enzyme, in fact, only appeared in 1993 (*98*).) In other early work, Fisher *et al.* (*99*) studied the poor substrate cefoxitin and obtained evidence that the lactamase-catalyzed reaction proceeds via an acyl-enzyme intermediate involving a serine residue. This conclusion was recently corroborated crystallographically (*100*).

The primary focus of the group, however, was always on *inhibitors* of β-lactamase. Clavulanic acid was isolated in 1976 by workers at Beecham (*101*), and soon thereafter, numerous additional inhibitors were discovered. The molecular basis of their action was far from clear, however, and the Knowles lab spent 8 years resolving