(1 H, d, J = 10 Hz, vinyl), 4.1 (1 H, m, br, allylic C*H*-O), 3.76 (2 H, td, J = 8, 4 Hz, CH_2OH), 2.3-1.5 (7 H, m), 1.17 (1 H, dd, J = 21, 9 Hz, one of ring CH_2), 1.00 (3 H, d, J = 7.1 Hz, Me).

The diol **27a** was protected as its bis(methoxymethyl) ether (**27b**) by the standard method as follows. To a solution of **27a** (10 mg, 0.064 mmol) in dry dichloromethane (1 mL) was added, via syringe, diisopropylethylamine (0.07 mL) and chloromethyl methyl ether (0.066 mL). The mixture was boiled under reflux for 5 h (monitored by TLC) and cooled to room temperature, and water (2 mL) was added. After the mixture was stirred for 5 min, the organic layer was separated and the aqueous layer was extracted with a further 5 mL of dichloromethane. The combined extracts were washed with water (2 × 10 mL), dried ($MgSO_4$), and evaporated to give **27b** (12.5 mg, 80%) which was judged sufficiently pure for the next step. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 5.90 (1 H, ddd, J = 10, 4.9, 2.3 Hz, vinyl), 5.73 (1 H, dt, J = 10, 1 Hz, vinyl), 4.76 and 4.63 (1 H each, ABq, J_{AB} = 6.9 Hz, allylic $-OCH_2O-$), 4.63 (2 H, s, primary $-OCH_2O-$), 3.86 (1 H, br t, J = 3.5 Hz, allylic CH-O), 3.63 (2 H, t, J = 6.3 Hz, CH_2O), 3.37 (3 H, s), 3.36 (3 H, s), 2.2-1.4 (5 H, m), 1.24 (1 H, dd, J = 21, 11 Hz, one of ring CH_2), 1.01 (3 H, d, J = 7 Hz, Me).

The crude protected diol **27b** (10.8 mg, 0.044 mmol) was dissolved in a 2:1 mixture of dichloromethane and methanol (3 mL) and cooled to -78 °C. Ozone-oxygen was bubbled through the solution for 2 min, and excess ozone was removed by bubbling argon through the mixture. Solvent was evaporated and replaced by methanol at 0 °C. Sodium borohydride (3.3 mg) was added, and the mixture was stirred and warmed to room temperature. After 30 min the mixture was taken up in ether (10 mL) and the solution washed with water (2 × 10 mL), dried ($MgSO_4$), and evaporated to yield the diol **28** which was purified by semipreparative HPLC (8.7 mg, 70%). IR ($CHCl_3$) ν_{max} 3580, 3400; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 4.76 and 4.70 (1 H each, ABq, J_{AB} = 8 Hz, secondary O-C*H*$_2$-OMe), 4.63 (2 H, s, primary, OC*H*$_2$OMe), 3.75-3.5 (6 H, 3 overlapping m, 3 × CH_2-O), 3.45 (3 H, s), 3.38 (3 H, s), 1.9-1.4 (8 H), 0.96 (3 H, d, J = 6.7 Hz, Me). Found: C, 55.60; H, 10.05. Calcd for $C_{13}H_{28}O_6$: C, 55.69; H, 10.07.

X-ray Structure Determination of Complex 8e. The complex was crystallized by addition of pentane to a solution of **8e** in chloroform-ether and setting aside at room temperature.

The crystals belong to the monoclinic space group $P2_1/c$ with a = 15.053 (5) Å, b = 8.327 (3) Å, c = 15.583 (3) Å, and β = 108.26 (2)°; molecular formula = $C_{20}H_{22}MoO_5$. All unique data with sing $\theta/\lambda \leq 0.54$ were collected and 1968 (79%) were judged observed ($|F_o| \geq 3\sigma(F_o)$).[1] Solution and refinement were routine, and the final discrepancy index is 0.070 for the 1968 observed reflections. All crystallographic calculations were done on a PRIME 850 computer operated by the Cornell Chemistry Computing Facility. Principal programs employed were the following: REDUCE and UNIQUE, data reduction programs by M. E. Leonowicz, Cornell University, 1978; MULTAN 78, a system of computer programs for the automatic solution of crystal structures from X-ray diffraction data (locally modified to perform all Fourier calculations including Patterson syntheses) written by P. Main, S. E. Hull, L. Lessinger, G. Germain, J. P. Declercq, and M. M. Woolfson, University of York, England, 1978; BLS78A, an anisotropic block diagonal least squares refinement written by K. Hirotsu and E. Arnold, Cornell University, 1980; PLUTO78, a crystallographic illustration program by W. D. S. Motherwell, Cambridge Crystallographic Data Centre, 1978; and BOND, a program to calculate molecular parameters and prepare tables written by K. Hirotsu, Cornell University, 1978. The structure is shown in Figure 2, numbering arbitrary. Bond distances are given in Table II and bond angles in Table III.

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Supplementary Material Available: Tables of fractional coordinates, thermal parameters (Table I) and torsion angles (Table IV) for **8e** (7 pages). Ordering information is given on any current masthead page.

Dynamic Transduction of Energy and Internal Equilibria in Enzymes: A Reexamination of Pyruvate Kinase†

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Abstract: The rate of interconversion of enzyme-substrate and enzyme-product complexes of pyruvate kinase was measured under equilibrium conditions using dynamic NMR methods. No evidence was found to support a significant contribution to the rate of catalysis by dynamic funneling of vibrational energy within the protein molecule. Furthermore, contrary to a previous report, the internal equilibrium constant between enzyme-substrate and enzyme-product complexes is different from unity. Serious reevaluation is necessary of the notion that enzymes in general match the free energies of bound intermediates.

An important goal of modern bioorganic chemistry is to understand how enzymes enormously enhance the rates of organic reactions.[1] In pursuit of this understanding, mechanisms for catalysis are often suggested that are not generally regarded as accessible to "normal" reactions in solution. Rather, these "special" mechanisms for enzymic catalysis are presumed to be uniquely accessible to macromolecules (i.e., enzymes). This paper concerns two suggested special mechanisms that have gained particular prominence recently.[2,3]

† Abbreviations: PK, pyruvate kinase; NMR, nuclear magnetic resonance spectroscopy; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; AMP, adenosine 5'-monophosphate; PEP, phosphoenolypyruvate; DTE, dynamic transduction of energy; EDTA, ethylenediaminetetraacetic acid; ES, enzyme-substrate complex; EP, enzyme-product complex.

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(1) Vibrational energy may behave nonergodically in proteins.[4] Mechanisms for the dynamic transduction of energy (DTE) may operate in protein catalysis to "funnel" vibrational energy released upon the binding of substrate into the active site; the protein molecule may prevent the dissipation of energy released upon the binding of the substrate "before it can be used for the enzymatic reaction".[2c]

(2) Matching of the free energies of enzyme-bound intermediates may be important for catalysis.[3] For example, it is commonly believed that "the equilibrium constant for the interconversion step approaches unity for an optimally evolved enzyme".[3b]

These suggestions raise anew an important question in bioorganic chemistry: *Is catalysis by enzymes fundamentally different from simpler catalysts in solution*? The two conjectures mentioned above assert that it is. This paper is concerned with further developing these two ideas theoretically and constructing explicit experimental tests for these special mechanisms in a single enzyme, pyruvate kinase, chosen as a model.

To experimentally examine the hypotheses regarding "dynamic transduction of energy" (DTE), such transduction as a mechanism for effecting catalysis must be clearly defined. We mean phenomena created by the protein macromolecule that perturb the distribution of vibrational energy from a normal Boltzmann distribution in an enzyme–substrate complex undergoing catalytic turnover, in a way that locally "heats" the bound substrate. In this mechanism, the desired reaction is facilitated *not* by altering the potential energy surface but rather by altering the populations of excited states in a fashion productive to the reaction.

Such a behavior is consistent with thermodynamic principles. Immediately after a substrate binds to an enzyme, until the vibrational energy "relaxes", thermodynamic principles do not require an equilibrium distribution of the thermal energy among the many vibrational modes in an enzyme–substrate (ES) complex. Thus, it is conceivable that an enzyme could store the energy released upon binding of the substrate in isolated vibrational modes and deliver that energy to a reactive bond of the substrate in the active site.[2c]

Such "nonergodic"[4] behavior, in which vibrational energy is *not* randomly distributed throughout all possible vibrational modes in a time rapid compared to the time required for reaction, admittedly has few precedents in organic chemistry in solution, although the very slow vibrational relaxation rates of certain pure liquids may be taken as models for such behavior.[5] Only rarely is nonrandom distribution of vibrational energy invoked to explain the reactivity of small molecules in the gas phase,[6] although uncovering such behavior is a major goal of physical chemists. Nevertheless, these considerations do not necessarily argue against the possibility that evolution may have selected enzymes that achieve catalysis by such a mechanism. In fact, the suggestion that enzymic catalysis is fundamentally different from catalysis in solution implies a denial of the relevance of organic models to enzymic reactions.[7]

The key to an experimental test of dynamic transduction of energy is the fact that it must occur rapidly following substrate binding, before the vibrational energy released is dissipated throughout all vibrational modes and before the system attains thermal equilibrium. Once in an equilibrium state, dynamic motions of the protein cannot increase reaction rates by a similar

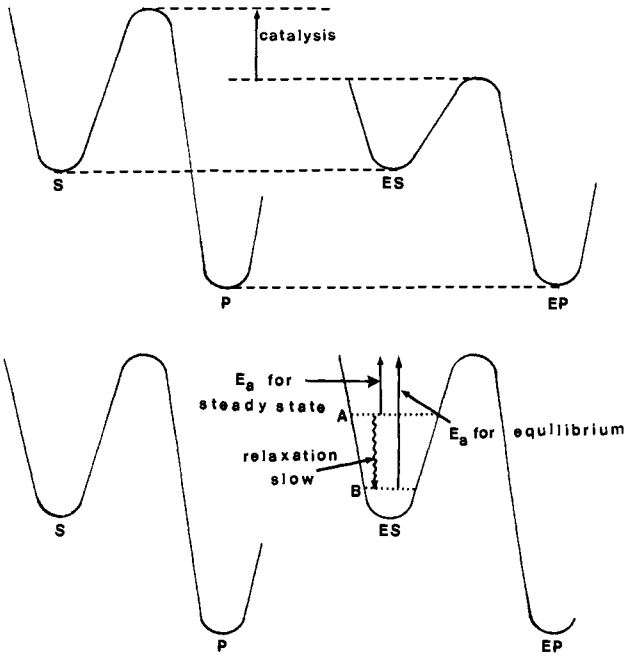

Figure 1. "Classical" mechanism for enzymic catalysis: (top potential energy surface) the enzyme alters the energy surface so as to make the barrier for converting ES to EP lower than the barrier for converting S to P. Under conditions of substrate saturation, the change in activation energy that corresponds to the enthalpy change associated with "catalysis" is the arrow in the top profile. In models for enzymic catalysis where dynamic transduction of energy (DTE) is important, the kinetically significant state of ES is vibrationally excited (labeled A in the lower potential energy surface). Many physical "pictures" can be associated with that excitation, e.g., dynamic coupled motion of the protein, vibrational energy stored in isolated vibrational modes. The macromolecular catalysts is presumed to act in a special way, slowing the rate of relaxation of vibrationally excited ES to the ground state (labeled B in the lower surface), or otherwise storing vibrational energy in "hot spots".[2b] In DTE models, catalysis can in the extreme be achieved by enzymes that do not alter the potential energy surface at all (as has been illustrated here).

mechanism. Although random fluctuations can provide activation energy to an enzyme-bound substrate by the same process that activation energy is acquired in normal reactions, under equilibrium conditions an enzyme cannot funnel random energy in a special way that manages to distort the energy of the bound substrates such that energized states are more populated than a normal Boltzmann distribution would allow under equilibrium conditions.

The fact that DTE mechanisms can only operate under nonequilibrium conditions provides a way to detect experimentally energy funneling as a mechanism for obtaining catalysis. We may compare the rate of interconversion of enzyme–substrate and enzyme–product complexes under nonequilibrium conditions (steady-state turnover) with the rate measured under equilibrium conditions. *If dynamic transduction of energy is important for catalysis, vibrationally relaxed enzyme–substrate complexes will not turn over with a fast enough rate to be able to account for the enzyme–substrate turnover rate at the steady state.* Conversely, if the rate of interconversion of vibrationally relaxed enzyme–substrate and enzyme–product complexes is as fast or faster than the turnover in the steady state, the *dynamic transduction of energy has no importance in enzymic catalysis.* This is illustrated in Figure 1.

Measuring the rate of interconversion of ES and EP complexes at equilibrium presents more experimental problems. Significantly, such interconversion rates are most easily measured when the internal equilibrium constant is close to unity, making NMR signals arising from enzyme-bound reactants and enzyme-bound products approximately of equal intensity. The rate of interconversion of these two complexes can then be measured by magnetization transfer or other dynamic NMR techniques.[8]

(3) (a) Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5631–5640. (b) Nageswara Rao, B. D.; Cohn, M. *J. Biol. Chem.* **1981**, *256*, 1716–1721. (c) Benner, S. A. *Experientia* **1982**, *38*, 633–636.

(4) Ergodic behavior means that a molecule is able to sample all vibrational and rotational states before it reacts, thus implying that vibrational energy is distributed over all modes of a system in an equilibrium (Boltzmann) fashion.

(5) For example, the vibrational relaxation time in liquid nitrogen is 1.5 ± 0.5 s at 78 K: Calaway, W. F.; Ewing, G. E. *Chem. Phys. Lett.* **1975**, *30*, 485–489.

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(7) Sinnott, M. L. *Chem. Brit.* **1979**, *15*, 293–297.

Direct observation of NMR signals arising from ES and EP complexes under equilibrium conditions was pioneered in part by Cohn and co-workers,[9a] who were among the first to suggest that internal equilibrium constants between enzyme-bound species were close to unity.[9] Pyruvate kinase, an enzyme explored in these studies, has become the paradigmatic example of an enzyme where the "internal equilibrium constant" (between enzyme-bound species) is approximately unity,[9a] despite an external equilibrium constant favoring ATP and pyruvate by approximately 4 orders of magnitude. Because of the importance of the enzyme in metabolism, suggesting that it may be "optimized" by evolutionary selection pressures to take advantage of special mechanisms for catalysis, and because the enzyme operates physiologically under nonequilibrium conditions, pyruvate kinase was chosen as the system for these studies.

Experimental Section

Pyruvate kinase (PK) was isolated from rabbit muscle by the method described by Tietz and Ochoa.[10] The enzyme was repeatedly recrystallized by the addition of crystalline ammonium sulfate to a solution of PK in 0.02 M imidazole buffer containing 1 mM EDTA at pH 7.0 and in some cases by preparative isoelectric focusing. PK was assayed spectrophotometrically by using a coupled assay with lactate dehydrogenase and NADH.[10] Fresh batches of enzyme had specific activities of approximately 400 units/mg, although, upon storage as crystals suspended in ammonium sulfate solution, the activity was found to decrease to 250 units/mg. The enzyme preparations were routinely checked by NMR for contaminating traces of compounds containing phosphorus.

ATP, ADP, sodium pyruvate, and phosphoenolpyruvate were purchased from Sigma and Boehringer Mannheim, and their purity was assured by phosphorus NMR. Tritiated ATP was purchased from New England Nuclear (31.3 Ci/mmol) and diluted with cold ATP.

All NMR experiments were run in 10-mm tubes, with 1–5 mM pyruvate kinase (based on active sites) in buffers containing Hepes (100–125 mM, pH 8.0), KCl (0.125 M), and $MgCl_2$ (7.83 mM). Spectra were obtained by using a Bruker 300-MHz superconducting NMR spectrometer operating with a broad-band probe at approximately 121 MHz. In simple pulse sequences, acquisition times were 0.5–1.0 s, with delays of 2–4 s between acquisitions. DANTE pulse sequences[8] consisted of a string of 35 1.2-μ pulses separated by delay times of 250 μs, followed by a 90° pulse and acquisition. Pulse sequences of this type deliver magnetization in discrete bands over narrow frequency ranges, determined by the offset frequency and the delay time. If the times are appropriately selected, such sequences invert the irradiated signal. In magnetization transfer experiments, a second delay time was introduced between the DANTE sequence and the acquisition.

The NMR spectrometer was operated under computer control. Alternating DANTE and normal pulse sequences were applied to the sample, and appropriate free induction decays were accumulated in two separate memories. By this technique, both DANTE and normal spectra could be collected for the same sample at the same time.

Extended irradiation using a DANTE pulse sequence leads to the "saturation" of the irradiated nucleus. This saturation can be transferred by chemical exchange to other nuclei, if the rate of chemical exchange is sufficiently fast compared to the rate of relaxation, measured to be on the order of a second for nuclei observed in this system. To detect this transfer, a string of 16 000 DANTE pulses 0.5 μs in duration were applied over a period of 1 s under several conditions. Accumulation of data was begun immediately at the end of the DANTE sequence. The extent to which magnetization is transferred from the irradiated peak to other peaks is a measure of the rate at which species are interconverting in the active site of the enzyme.[8]

Temperatures were maintained constant, depending on the run, between 5 and 20 °C. In many of the NMR experiments, we reproduced the conditions reported by Nageswara Rao et al.[9a] as precisely as possible, including using the identical buffers at identical concentrations, identical substrate concentrations, identical temperatures, and identical NMR pulse parameters.

Pyruvate was assayed using lactate dehydrogenase to catalyze the oxidation of NADH in the presence of pyruvate. Similarly, ATP was estimated by using a coupled assay consisting of hexokinase and glucose-6-phosphate dehydrogenase, observing spectrophotometrically the appearance of NADH at 340 nm.

Quench studies were performed on solutions of the enzyme–substrate complex in which the ATP was labeled with tritium. The concentrations of buffer, ATP, and sodium pyruvate were identical with those used to prepare the NMR solutions, and the enzyme concentration was varied between 4.7 and 0.002 mM (active sites). Mixtures were prepared and equilibrated (10 min) at 0 °C. The mixtures were then quenched at 0 °C either by rapid addition of EDTA (0.2 M final concentration) followed 5 min later by immersion in boiling water (5 min) or by rapid addition of trichloroacetic acid (15% final concentration) followed 2 min later by the addition of excess saturated sodium bicarbonate. Cold carriers (ATP and ADP) were then added, the precipitated protein was removed by centrifugation, and the supernatants were chromatographed on thin-layer silica gel plates (E. Merck 60 F 254 analytical plates; *n*-propyl alcohol–ammonia–water 55:35:10 as eluant). The position of the compounds was determined by their absorbances of ultraviolet light, and silica gel was scraped from appropriate sections of the plate (along with control sections of the plate), extracted overnight with 0.5 mL of water, and counted in 4 mL of Aquasol.

Analysis by thin-layer chromatography showed 2.5% contamination of the stock tritiated ATP by tritiated ADP. There was no hydrolysis of ATP during either the quench or the chromatography. To assess the extent of enzyme-catalyzed hydrolysis of ATP at high concentrations of PK, mixtures were quenched after incubation for 1 h at 0 °C.

Results

The resonances of the γ phosphorus of ATP and the β phosphorus of ADP are not resolved in NMR spectra of these molecules bound in the active site of pyruvate kinase. They give rise to a signal that we shall refer to as peak B (Figure 2). Irradiation at peak B should affect the intensity of resonances arising from phosphorus atoms that are in chemical exchange with these two atoms, specifically, the β phosphorus of ATP (peak D in Figure 2, in exchange with the β phosphorus of ADP) and the phosphorus of PEP (peak A in Figure 2, in exchange with the γ phosphorus of ATP). In fact, irradiation affects the intensity of peak D but not the intensity of peak A (the resonance assigned to PEP by Nageswara Rao et al.[9a]). DANTE pulse sequences lasting 8 ms produced only partial inversion of peak B under conditions where dynamic exchange was occurring (Figure 2A,B). The intensity of peak D was diminished only slightly. However, in the presence of excess EDTA (which chelates the Mg^{2+} required for the enzyme to catalyze the turnover), the irradiated peak was completely inverted (Figure 2C), without noticeable diminution of the signal assigned to the β P of ATP (peak D).

The incomplete inversion of the irradiated peak under conditions where the reaction is occurring suggests that a dynamic process is removing through chemical exchange some magnetization that is being introduced by the DANTE irradiation sequence. The slight diminution of intensity of the resonance arising from the β P of ATP (peak D) is consistent with there being only a small amount of ADP contributing to the irradiated resonance, but the diminution is too small to be the basis for further conclusions. However, these data are consistent with chemical interconversion of ATP and ADP occurring with a half-life on the order of the length of the DANTE sequence (8 ms) and an internal equilibrium favoring ATP. However, they seem inconsistent with the assignments of Nageswara Rao et al. and an internal equilibrium constant of approximately unity, as one would expect corresponding loss of intensity of *both* peaks A and D upon irradiation of B. Similar experiments in which peak A or peak D was irradiated led to similar conclusions.

Continued irradiation (1 s) of peak B leads to more substantial loss of intensity of peak D. The extent of the disappearence depends on the concentrations of enzyme and total pyruvate in a fashion that appears to correlate with the amount of ternary complex. Two spectra (Figure 2D and E), at enzyme concentrations of 1.2 and 2.2 mM, show this dependence; at the higher concentration of enzyme, where more of the substrate is expected to be in the ternary complex undergoing chemical exchange, peak D is smaller. Similarly, in data not shown, addition of excess pyruvate (50 mM) leads to greater transfer of magnetization. These experiments demonstrate that the disappearance of intensity in peak D upon DANTE irradiation at peak B is not due to

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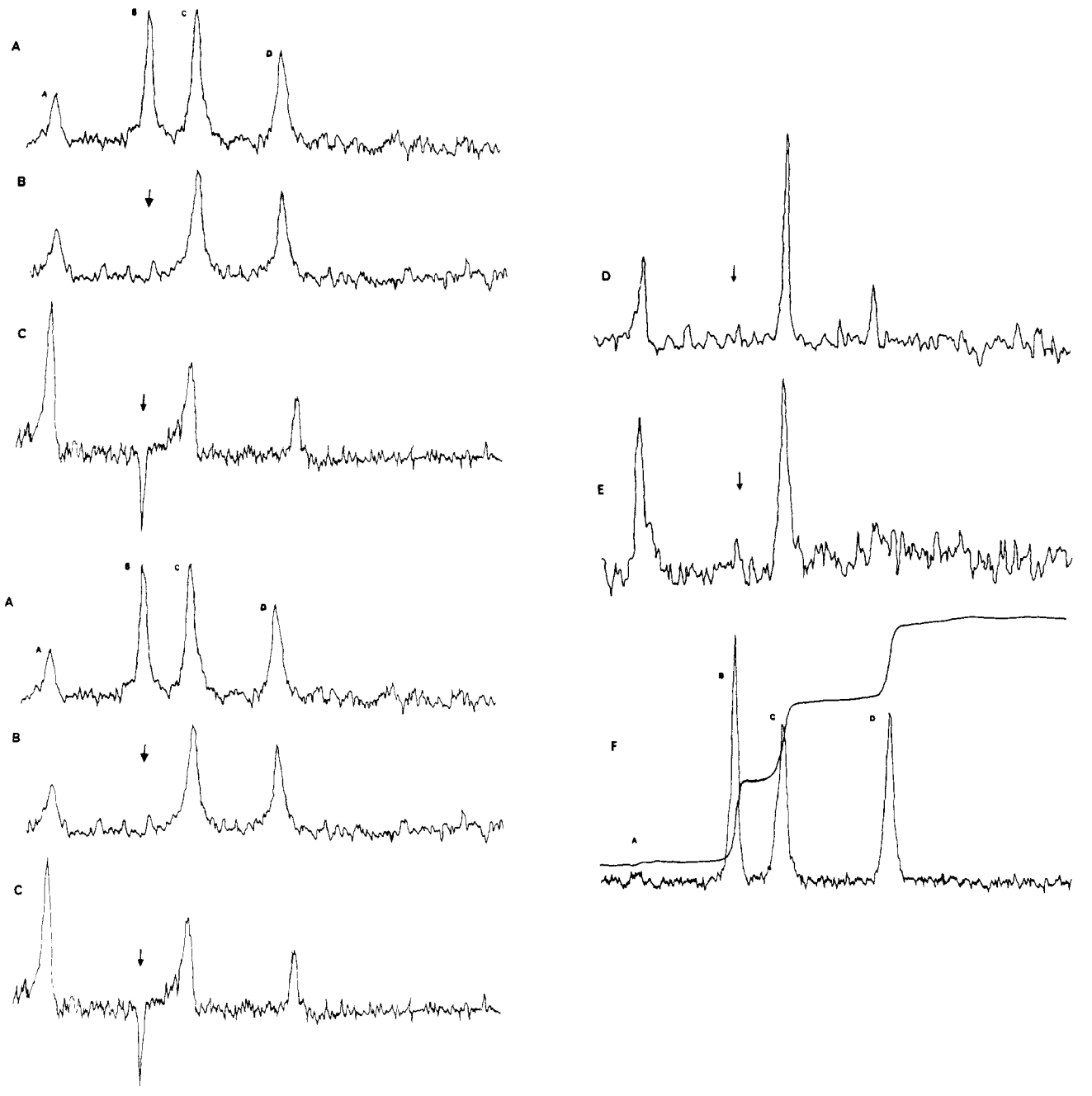

Figure 2. Phosphorus-31 NMR spectra showing enzyme-substrate and enzyme-product complexes at equilibrium. (a) Pyruvate kinase (3.7 mM active sites), 3.2 mM ADP and PEP, under conditions of Nageswara Rao et al.[9a] (b) Same as A with DANTE irradiation (Total time, 8 ms) at the position of the arrow, under conditions of chemical exchange (resonance B exchanging with resonance D and, allegedly, with resonance A). (c) Same as B but with EDTA added in molar excess over Mg^{+}; equilibrium is presumably "frozen" on the active site,[9a] with chemical exchange no longer occurring due to removal of essential Mg^{2+}, irradiated at the position of the arrow. (d) Pyruvate kinase (1.2 mM), ADP (1.2 mM), and PEP (1.2 mM) with DANTE irradiation (total time, 1 s) at the position of the arrow. Note the loss of intensity of the resonance assigned to the β P of ATP (peak D) but not of the resonance supposedly arising from PEP (peak A). (e) Pyruvate kinase (2.2 mM), ADP (2.2 mM), and PEP (2.2 mM) with DANTE irradiation (total time, 1 s) at the position of the arrow. Note the greater loss of intensity of peak D (β P of ATP) but not of peak A (supposedly arising from PEP). (f) Pyruvate kinase (5 mM), PEP, and ADP, incubated for 5 min, with reaction and then quenched by addition of excess EDTA; data accumulation for 7 h under conditions where no inorganic phosphate was produced.

intramolecular transfer of magnetization by processes other than chemical exchange. Significantly, *this irradiation does not substantially affect the intensity of peak A*, previously assigned to PEP (along with inorganic phosphate and AMP).

In these experiments, the degree of diminution of the area of a resonance arising from an atom in chemical exchange with an irradiated species can be far in excess of the amount of the irradiated species at equilibrium, if exchange occurs many times in the course of the irradiation. The only requirement is that the rate of exchange be greater than the rate of relaxation of the nuclei (measured to be on the order of a second in these systems). Therefore, these results suggest strongly that ATP and ADP interconvert at equilibrium many times in 1 s, suggesting a lower limit of 10 s^{-1} for the rate of interconversion and an internal equilibrium constant of approximately 10:1.

Because this internal equilibrium constant is substantially different from the previously estimated value of unity,[9a] the rates of interconversion of bound complexes under equilibrium conditions can only be estimated. They are consistent with estimates by Nageswara Rao et al.[9a] based on changes in line widths upon addition of EDTA, although we recognize that interpretation of line widths is complicated by other factors. As these rate constants are not significantly different from the steady-state turnover rate constant of approximately 60 s^{-1} for pyruvate kinase under the

conditions used in our NMR measurements, we have found no evidence that supports a substantial role for dynamic transduction of energy in catalysis by this enzyme.

However, surprisingly, our data also directly challenge the belief often expressed by bioorganic chemists (including ourselves) that enzymes generally match the free energies of bound species.[3]

The most striking feature obvious from NMR studies on pyruvate kinase under the conditions described by Nageswara Rao et al.[9a] is the existence of an activity that slowly decreases the NMR signal associated with ATP and produces a new peak in the NMR approximately 9.6 ppm downfield from the peak assigned by Nageswara Rao et al. to the γ P of ATP. This activity is apparently not diminished even by repeated (6X) recrystallization nor by preparative isoelectric focusing of the enzyme, suggesting that it is an inherent activity of pure pyruvate kinase.[11] We estimate that the first-order rate constant of hydrolysis of bound ATP in the absence of pyruvate is between 10^6 and 10^7 times slower than k_{cat} for the phosphoryl-transfer reaction.

The new signal appears at the expense of ATP and concomitantly leads to an increase in intensity of peak A to enzyme-bound ADP and PEP. "Active phosphate" (ATP + PEP), assayed using hexokinase and glucose-6-phosphate dehydrogenase in the presence of pyruvate kinase, declines as this reaction proceeds. Finally, the new peak has the same chemical shift as added inorganic phosphate. Together, these facts indicate that under the conditions of the NMR experiments, ATP is being slowly hydrolyzed to form ADP and inorganic phosphate.

This hydrolysis occurs to a significant extent during the time required to obtain satisfactory signal-to-noise ratios in the system. Furthermore, the chemical shift of the putative inorganic phosphate is the same as that of peak A, assigned by Nageawara Rao et al.[9a] predominantly to enzyme-bound PEP (and also to inorganic phosphate and AMP. The hydrolysis reaction is slower at 5 °C than at 20 °C, is slowed in the presence of increased concentrations of pyruvate, and is completely inhibited in the presence of EDTA when in molar cells over total magnesium, presumably because Mg^{2+} is required for the enzymatic hydrolysis reaction.

Nageswara Rao et al.[9a] also observed this hydrolysis and also realized that the resonance of inorganic phosphate was superimposed on that for PEP. Nevertheless, by extrapolation, they estimated the fraction of the peak arising from PEP and the fraction arising from inorganic phosphate (and AMP) and used this estimate to calculate that the internal equilibrium constant between the two ternary complexes, enzyme–PEP–ADP and enzyme–pyruvate–ATP, is approximately unity.

We believe this estimate needs adjustment in light of five facts:

(1) Under controlled conditions, including conditions identical with those used in the original experiments,[9a] no signal can be found that might be assigned to enzyme-bound PEP, after extrapolation of the intensity of the relevant resonance to zero time, an extrapolation designed to subtract the signal arising from inorganic phosphate produced by the slow hydrolysis of ATP. These results set an upper limit on the amount of enzyme-bound PEP at equilibrium and thus indicate that the internal equilibrium constant in this enzyme is greater than 1:1 in favor of ATP and pyruvate.

(2) DANTE pulse sequences that produce a partial inversion of the resonance assigned to the γ P of ATP (peak B) do not alter the intensity of the resonance assigned to enzyme-bound PEP (peak A). Given the esitmate[9a] for the rate of exchange between these two species of 10^2 s^{-1}, the assignment of a significant portion of peak A as PEP is inconsistent with these data. The extent to which these DANTE sequences diminish the intensity of peak D is also inconsistent with more than 10% of peak A being enzyme-bound PEP.

(3) Complete saturation for 1 s of peak B does not alter the intensity of peak A, even though sufficient saturation is transferred during the pulse sequence to the β phosphorus of ATP to almost completely eliminate peak D (Figure 2D and E).

(11) Professor Albert Mildvan has suggested that this activity may be reduced by further chromatography.

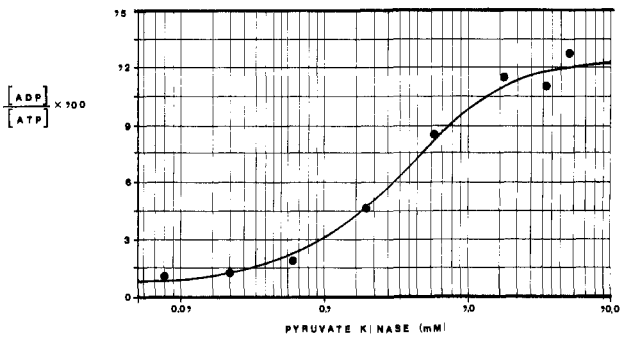

Figure 3. Plot of ADP/ATP as a function of concentration of pyruvate kinase in equilibrium quench experiments. The data were corrected for a small (2.5%) contamination of stock tritiated ATP by tritiated ADP but were not corrected for the slow hydrolysis of ATP observed under conditions of high enzyme concentration. Thus, the titration curve is slightly distorted on the right-hand side, indicating a ratio of ADP/ATP too high by approximately 20%.

(4) Nageswara Rao et al.[9a] noted that addition of EDTA in molar excess over Mg^{2+} "froze" the internal equilibrium, presumably by removing the Mg^{2+} needed for catalysis, without causing dissociation of enzyme–substrate complexes. As EDTA at these concentrations completely inhibits the hydrolysis reaction, and as the internal equilibrium is attained rapidly (less than 1 s), we added substrates to the enzyme under conditions identical with those used by Nageswara Rao et al.,[9a] allowed the mixture to incubate at 5 °C for 5 min, added molar excess of EDTA, and then accumulated an NMR spectrum for 5 h without the hydrolysis reaction interfering. The spectrum is shown in Figure 2F. Only a very small signal is apparent, having a chemical shift similar to that assigned by Nageswara Rao et al.[9a] to enzyme–bound PEP. The excellent signal-to-noise ratio permits the estimation of an internal equilibrium constant no less than 13:1 in favor of enzyme–ATP–pyruvate.

(5) The equilibrium ratios between ATP and ADP were measured in quench experiments as a function of the concentration of pyruvate kinase (Figure 3). The plot is a "titration curve" representing the titration of the substrate onto the active site as the enzyme increases in concentration. As the amounts of PEP and ADP in solution are negligible, these species in the quench mixture must arise from these species enzyme-bound. The point of inflection in the titration curve is at an enzyme concentration of 400 μM, approximately the same as the dissocation constant for the substrate. Although it is difficult to obtain concentrations of enzyme high enough to bind over 99% of nucleotide, the apparent plateau occurs where it should give a "half-titration" point at 400 μM. These data are consistent with an internal equilibrium constant of approximately 10:1 in favor of enzyme–ATP–pyruvate.[12,13]

Discussion

Consider two ways in which an enzyme might make a reaction go faster.

First, the enzyme might alter the energy surface of the reaction, for example, by making energetically favorable or unfavorable interactions with structures that are intermediate along the reaction coordinate. This is, of course, the "classical" mechanism for achieving catalysis. The enthalpic barrier for the reaction is lower in the surface altered by enzyme–reactant interactions, and so the rate of the reaction is faster.

In this model, the activation energy needed to surmount activation barriers is still obtained by "normal" processes. In the vibrationally excited states, the enzyme–substrates (ES) complex

(12) These experiments were repeated under several conditions presumed to more closely approximate physiological conditions (pH 7.2 and 7.4, 37 °C). In all cases, the equilibrium constant remained approximately 10:1.

(13) These equilibrium quench experiments are different from the kinetic quench experiments pioneered by Rose and co-workers: Rose, I. A.; O'Connell, E. L.; Litwin, S.; Bar Tana, J. *J. Biol. Chem.* **1974**, *249*, 5163–5168.

is populated in a Boltzmann distribution, where energy is rapidly (compared to the rate of reaction) transferred between vibrational, translational, and rotational modes by the random collision of molecules. Complexes having the appropriate energy react and these are rapidly replaced in the distribution from lower states with energy obtained from other molecules by random collisions. By this classical mechanism, ES complexes obtain activation energy by processes found in conventional catalysis. In the process, the protein may move, but these movements are important only because they accommodate intermediates and transition states having differing structures or permit the entry and exit of compounds from the active site.[14]

Second, an enzyme might achieve catalysis without altering the height of barriers along an energy surface but rather by altering the population of vibrationally excited enzyme–substrate states on the same energy surface. This mechanism requires the "funneling" of energy and is associated with several mental pictures where the dynamic motions of the protein itself speed reactants over enthalpic barriers to products. For example the protein might move to bump into the substrate, "kicking" it over an enthalpic barrier. In another picture, vibrational energy (originating from the enthalpy associated with enzyme–substrate binding) is funneled into a reactive bond, via specific, restricted couplings of vibrational modes in the protein that pass along the vibrational energy without dissipating it to solution. Thus, the reactive bond is "heated" to a high temperature, the population of vibrationally excited states is increased, and the reaction goes faster. Such pictures involve *vectorial processes*. Peptide units and energy move in specific directions. Thermodynamically, work is being done, and for work to be done, thermodynamics requires that a system be out of equilibrium.

Our experiments distinguish between these two models. The rate an enzyme–substrate (ES) complex yields an enzyme–product (EP) complex is compared under two circumstances, at equilibrium, and under conditions of steady-state turnover. Under equilibrium conditions, the population of vibrationally excited states must be Boltzmann, and DTE mechanisms cannot be operative. Under the nonequlibrium conditions of the steady state, a Boltzmann distribution of excited states need not exist, and DTE mechanisms might be operative. Experimentally, we find the rates under the two conditions to be the same. Therefore, DTE mechanisms are not important for catalysis in pyruvate kinase.

An alternative perspective is diagramed in Figure 1. In the classical mechanism, the barrier to interconversion of ES and EP is lower than the barrier for converting S and P, because enzyme "differentially binds" S, P, and the transition state between them so as to alter the free energy profile. The kinetically significant ES complex is in its lowest vibrational state.

In contrast, DTE mechanisms postulate (in the extreme) that the enzyme does not alter the energy surface. Rather, catalysis is achieved by the enzyme, in a dynamic process, by preventing the ES complex from vibrationally relaxing from an excited state (labeled A in Figure 1) into the ground state (labeled B). Molecules in the excited state surmount the barrier more easily because they already have a substantial fraction of the needed activation energy *in vibrational form.*

Our experiment can be viewed as an attempt to observe the relaxed complex B by preparing the ES complex labeled A (Figure 1) under equilibrium conditions and waiting for the system to come to vibrational equilibrium. The waiting time of approximately 1 h is 10^6 times longer than the half-life of the ES complex under steady-state conditions and much longer than the enzyme needs to hold vibrational energy out of equilibrium to exploit DTE mechanisms. However, no evidence of a vibrationally relaxed state other than the kinetically significant state can be found.

Status of "Dynamic Energy Transduction". Our estimate for the rate of interconversion of enzyme-bound ternary complexes

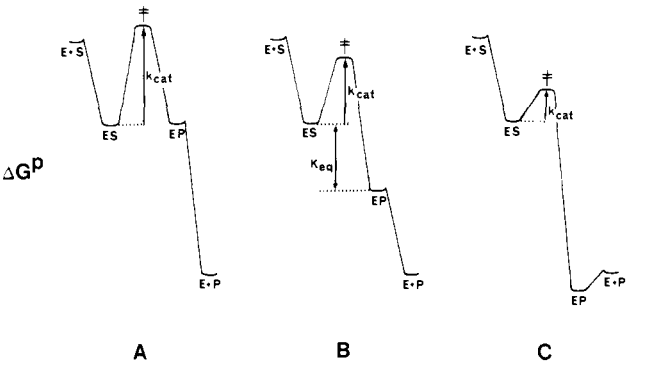

Figure 4. Plots of $\Delta G^p = \Delta G^\circ + RT \ln [P]/[S]$ vs. reaction coordinate for a number of hypothetical enzymatic free energy profiles. Given the assumptions that (a) the enzyme operates physiologically with a free energy drop between substrate and products, (b) a linear free energy relationship connects the rate constant associated with the catalytic step (ES to EP) with the equilibrium constant between ES and EP, and (c) that the enzyme has not already evolved so as to make the catalytic step not rate limiting, then the fastest flux will be with a descending staircase free energy profile (profile B). An enzyme with an internal equilibrium constant of unity (profile A) can be made faster by lowering the energy of the EP complex to give profile B. Further lowering of the energy of the EP complex (profile C) does not produce a still faster enzyme, due to product inhibition. The precise magnitude of the internal equilibrium constant depends only on the physiological free energy drop and the nature of the linear free energy relationship. Given the assumptions of Albery and Knowles[3a] concerning the evolution of enzymes, profile B is the evolutionarily optimal one. For enzymes operating physiologically at equilibrium, the descending staircase produces an internal equilibrium constant of unity as a special case.

in pyruvate kinase at equilibrium, based on magnetization-transfer experiments, indicates that enzyme–substrate complexes at vibrational equilibrium are kinetically competent to sustain the turnover observed under steady-state conditions. Because the internal equilibrium constant for pyruvate kinase is not unity, these estimates contain a substantial degree of error. However, even assuming a large error in measurement, our results are inconsistent with nonergodic[4] behavior being a significant mechanism for enzymic catalysis of major importance. At least in this case in this respect, it seems as if enzymes are not qualitatively different from ordinary catalysts.

Status of "Matched Internal Thermodynamics". The measured "internal" equilibrium constant for pyruvate kinase is 10:1 to 15:1 in favor of the enzyme–ATP–pyruvate complex. This is an order of magnitude differnt from the 1:1 ratio reported by Cohn,[9a] although the equilibrium constant between bound substrates is still nearer to unity than the equilibrium constant between the same species in solution. While it appears true that this enzyme binds substrates so as to make their energies more closely matched than their energies in solution, this enzyme cannot be said to "match" the internal thermodynamics in this system. In fact, given the overall equilibrium constant between the free species of 6500, the internal equilibrium constant reflects approximately one-third of the free energy difference intrinsic in the free reactants and free products. Given an appropriate choice of standard states, the free energy profile of pyruvate kinase more closely resembles a descending staircase than a pair of bound states with matched energies (Figure 4).

This order of magnitude deviation has major significance in the theoretical understanding of free energy profiles of enzyme-catalyzed processes. At issue are two specific types of profile, which are represented in Figure 4 as the "matched internal free energy profile", and the "descending staircase" profile. The matched profile has often been assumed to be the more common free energy profile,[3,15] judging from empirical data collected on internal free energies,[9] in contrast to the descending staircase model. This assumption needs reevaluation.

(14) It is clear that, because of their size, macromolecular catalysts *must* move to permit these functions to be executed. Whether this is viewed positively (i.e., as a special advantage of macromolecular catalysts) or neutrally (i.e., something that has to happen or else the size of an enzyme would obstruct catalysis) is unclear at this point.

(15) Cook, P. F.; Cleland, W. W. *Biochemistry* **1981**, *20*, 1807–1816.

We note that the relative positions of the external states on a free energy plot depend on an arbitrarily chosen standard state. They move in relation to the enzyme-bound states depending on concentrations of free species. However, we may define G^P as the *physiological* free energy of the reaction catalyzed by any particular enzyme, given by the expression

$$\Delta G^P = \Delta G^\circ + RT \ln [P]/[S] \quad (1)$$

where [P] and [S] are the physiological concentrations of product and substrate, respectively.

We propose that the kinetically optimal free energy profile for an enzyme is one in which the drop in energies of the ES and EP states reflect some, but not all, of the free energy drop between reactants and substrates under physiological conditions.

A descending staircase free energy profile can be shown to be the profile that gives the fastest turnover of substrate to product under physiological conditions, provided that the rate of the microscopic catalytic step is related to the microscopic internal equilibrium constant.[3a] Relationships between rate constants and corresponding equilibrium constants are well-known in chemistry, as "linear free energy relationships" having the form

$$\log k = C + \beta \log K_{eq} \quad (2)$$

In pictures (Figure 4), an enzyme catalyzing a reaction having a physiological free energy drop ΔG^P, and having internal internal equilibrium constant of unity, can be made faster by lowering the energy of the EP complex with respect to the ES complex and lowering in the process the energy of the transition state in accordance with the linear free energy relationship.

In principle, the rate of the catalytic step can be made faster and faster by continuing to lower the free energy of the EP complex. However, at some point, further lowering of the energy of the EP complex, while making the rate constant for the microscopic catalytic step faster, will no longer increase the overall flux through the enzyme. This is because as the EP complex approaches the free energy of the E + P state, the off step will become rate limiting. In other language, at the physiological concentrations of the product, there will be substantial product inhibition.

We have previously noted[16] that the often-cited theoretical derivation of matched internal thermodynamics, provided by Albery and Knowles,[3a] is not inconsistent with this view. Although widely interpreted as supporting matching of the energies of bound intermediates under all conditions,[15,3b] the equations that are used by Albery and Knowles to derive "matched internal thermodynamics" are, in fact, different from those that govern the catalytic efficiency of enzymes under all circumstances.[17] Some of the limitations of the algebra in ref 3a were discussed by Chin.[18]

Indeed, the assumptions and formalism used by Albery and Knowles lead to a derivation of a descending staircase as the catalytically optimal free energy profile, with the precise magnitude of the internal equilibrium constant dependent only on the ΔG^P and on the magnitude of β. Thus, "matched internal thermodynamics" is expected to occur only in enzymes that catalyze steps that physiologically are close to equilibrium. In these cases, matched internal thermodynamics are simply a special case of a descending staircase, where the top and the bottom of the staircase are at the same level. The prediction base on this model is that enzymes other than pyruvate kinase that operate physiologically under nonequilibrium conditions will also not display matched internal thermodynamics. In fructosebisphosphatase as an example, this indeed seems to be the case.[19]

We have found evidence against nonergodic behavior in only a single enzyme, pyruvate kinase. Nevertheless, our approach is generally applicable, and our results on this system shed doubt on the importance of these "special" mechanisms unique to macromolecular catalysts in other enzymes as well. Similarly, our results with pyruvate kinase suggest that the "principle" of "matched internal thermodynamics" is in fact not a principle. If further studies on other enzymes find more examples of "descending staircase" free energy profiles,[20] they will constitute further evidence that "normal" physical organic principles can explain the detailed internal thermodynamic of enzymatic reactions.

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(16) Nambiar, K. P.; Stauffer, D. M.; Kolodziej, P. A.; Benner, S. A. *J. Am. Chem. Soc.* **1983**, *105*, 5886–5890.

(17) Specifically, these authors use a narrowly defined V/K function to describe enzymic efficiency in their differentiation seeking an optimum internal equilibrium constant, as opposed to a more complete efficiency function that includes all microscopic rate constants.

(18) Chin, J. *J. Am. Chem. Soc.* **1983**, *105*, 6502–6503.

(19) Rahil, J. F.; de Maine, M. M.; Benkovic, S. J. *Biochemistry* **1982**, *21*, 3358–3363.

(20) It should be noted that data collected on the energies of ES and EP complexes in dehydrogenases[16] also are consistent with the "descending staircase" model.