Internal Thermodynamics of Enzymes Determined by Equilibrium Quench: Values of K_{int} for Enolase and Creatine Kinase†

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ABSTRACT: The equilibrium constant (K_{int}) for the *enzyme-bound* substrate and product of a one substrate/one product enzyme (enolase) and for those of a two substrate/two product enzyme (creatine kinase) have been determined. The values of K_{int} were determined by the rapid quenching of equilibrium mixtures of enzyme and radiolabeled substrate and product, under conditions where all of the marker substrate and product are bound. The scope and limitations of this method are discussed. Values of K_{int} have been collected from the literature, and it is shown that these data are consistent with the theory for kinetically optimized enzymes that is developed in the preceding paper.

In the preceding paper, we have described how the energetics of enzyme-catalyzed reactions may change so as to optimize catalytic effectiveness. The kinetic characteristics of catalytically optimal enzymes are defined by these proposals, and our predictions can be compared with the behavior of real systems to test the validity of the theory. For example, the value of the internal equilibrium constant K_{int} (the equilibrium constant for the interconversion of enzyme-bound substrates and products) is a property of a catalytically optimized enzyme that depends on the in vivo conditions under which the enzyme evolved. In this paper, we describe the direct measurement of the value of K_{int} for two enzymes, enolase and creatine kinase. The significance of these measurements, together with the available data from the literature, is then discussed in terms of the theory developed in the previous paper (Burbaum et al., 1989).

To obtain K_{int}, the relative concentrations of productive enzyme/substrate and enzyme/product complexes must be determined. Four methods of measurement have been used to achieve this end: NMR spectroscopy, kinetic analysis, steady-state quench experiments, and equilibrium quench measurements. To determine K_{int} by NMR spectroscopy, the enzyme concentration must be large enough to ensure that its substrates and products are more or less fully bound. Because of this requirement, ^{1}H NMR is of little utility, since substrate and product resonances are generally difficult to distinguish from the high background of ^{1}H resonances from the protein. In principle, ^{13}C NMR can be used, both because the chemical shifts are large and because the selectivity for substrate resonances can be improved by specific isotopic enrichment. For large enzymes, however, the protein resonances are still troublesome, and ^{13}C NMR spectroscopy has not yet found widespread use in the determination of K_{int} [*pace*, however, Jaffe and Markham (1987)]. The nucleus that has been most frequently exploited for the measurement of K_{int} values is ^{31}P. Limitations in the measurement of K_{int} by ^{31}P NMR, however, do exist. Because enzyme substrates that contain ^{31}P are mostly phosphorus(V) esters or anhydrides, chemical shift differences between bound substrates and products are often small. In addition, the solubility of most enzymes limits the concentration of liganded enzyme complexes and therefore the sensitivity of the measurement. Finally, the rate constants of enzymatic processes often lie in the range of the frequency differences between ^{31}P NMR signals of substrate and product (10–1000 Hz), and only a single resonance may be observed. As a result of these constraints, measurement of K_{int} by ^{31}P NMR has been applied almost exclusively to enzymes that cleave either the α,β or the β,γ phosphoanhydride bond of nucleoside triphophates, because the central (β) phosphorus has a resonance frequency that is well clear of other phosphorus nuclei. By use of this approach, the values of K_{int} for a number of phosphotransfer enzymes have been determined, largely by Cohn and her collaborators (see the references to Table III).

Kinetic data may provide another route to the determination of K_{int}. Usually, both steady-state and pre-steady-state kinetic measurements of the enzyme-catalyzed reaction in both directions are required, and results from kinetic analysis are somewhat less palatable than those from NMR, because K_{int} is measured as a ratio of rate constants rather than as a ratio of concentrations. If the kinetic scheme were clear and all the rate measurements equally precise, of course, the two methods would have similar standing. But such clarity is rarely found, because it is usually easier to assign a structure and a concentration to a particular species than it is to assign a rate constant to a particular process. While most of the known values of K_{int} have been determined from NMR or from kinetic measurements, neither of these methods has the generality that would allow the routine determination of K_{int} for any enzyme.

A third method that has been employed to measure K_{int} is the steady-state quench experiment. Here, the target enzyme is mixed rapidly with radiolabeled substrate, the reaction is allowed to reach the steady state, and the mixture is then quenched either with a denaturant or with excess unlabeled substrate at different times after mixing. In favorable cases, the concentrations of the active enzyme complexes with substrate and with product in the steady state can be measured by extrapolation to zero time. These concentrations, however, represent the concentrations of the two complexes in the steady state, not at equilibrium. The internal equilibrium constant, K_{int}, will be measured only if the active enzyme complexes are at equilibrium in the steady state. This can occur if the complexes interconvert rapidly relative to other processes during turnover. As illustrated in Figure 1, if reaction of E·S over transition state 2 is matched to product release from E·P over transition state 3, then, even when K_{int} is large, the

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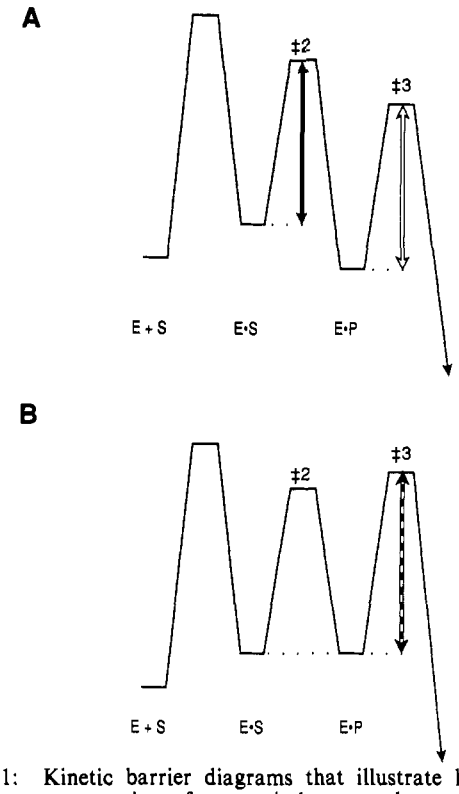


FIGURE 1: Kinetic barrier diagrams that illustrate how equal steady-state concentrations of enzyme/substrate and enzyme/product complexes may (B) or may not (A) reflect a value for the internal thermodynamics (K_{int}) of unity. The nature of kinetic barrier diagrams is described in Appendix I of the preceding paper (Burbaum et al., 1989).

steady-state concentrations of E·S and E·P will be nearly equal. Steady-state quench experiments therefore provide important information about the steady-state distribution of enzyme forms, but the data by themselves do not measure the value of K_{int}. In determining the catalytic optimality of an enzyme, however, this distribution can be as important as the value of K_{int}. This point is discussed in more detail later.

Finally, the value of K_{int} can be established from equilibrium quench experiments. These measurements are simpler in concept than the steady-state quench experiments discussed above, because the enzyme and its substrates and products are at equilibrium at the time of quenching. The only requirements (which are discussed under Theory), are that the substrates and products be completely and productively bound to the enzyme and that quenching be faster than turnover. An early example of the use of this approach is the determination of K_{int} for phosphoglucomutase by Ray and Long (1976a,b). In the present work, we have aimed to simplify the experimental protocol used by these workers and to explore the generality of the approach for different types of enzyme systems.

Experimental Procedures

Materials

Phosphoenol[1-^{14}C]pyruvate was synthesized from 3-phospho-(*R*)-[1-^{14}C]glycerate according to the method of Cohn et al. (1981). The labeled phosphoglycerate was prepared from ribulose 1,5-bisphosphate and sodium [^{14}C]bicarbonate (Addadi et al., 1983) by Dr. W. J. Guilford. The labeled phosphoenolpyruvate had a specific radioactivity of 25 mCi/mmol and was stored at −20 °C in ethanol–water (5% v/v). Immediately before use, the phosphoenol[1-^{14}C]pyruvate was freeze-dried and resuspended in water to a concentration of 10^5 dpm/μL. [8-^{14}C]Adenosine 5′-triphosphate was from Amersham (Arlington Heights, IL) and had a specific activity of 51 mCi/mmol. This material was purified before use as follows. A portion (10 μCi) was freeze-dried to remove ethanol, and the residue was purified by HPLC[1] using conditions identical with those for the analysis of a quench mixture (see below). Fractions (0.2 mL) containing more than 2×10^6 dpm/mL [8-^{14}C]ATP were pooled. To remove the phosphate contained in the elution buffer, this pool was diluted 10-fold with water and then loaded onto a column (1 mL) of DE-52 cellulose (Sigma, St. Louis, MO) equilibrated with 20 mM triethylammonium bicarbonate buffer, pH 8.0. The column was eluted with a linear gradient (20–200 mM, 100 mL plus 100 mL) of the same buffer. Fractions containing radioactivity were pooled into a silanized 100-mL pear-shaped flask. The solution and buffer were removed by rotary evaporation under reduced pressure, with repeated additions of isopropyl alcohol (Aldrich Gold Label, 99+%, Aldrich Chemical Co., Milwaukee, WI). The radioactive ATP was then dissolved in a small amount of ethanol–water (5% v/v) and was stored in several portions at −20 °C. Immediately before use, the solution of ATP was freeze-dried to remove ethanol and then redissolved in aqueous EDTA (0.5 mM).

Imidazole was recrystallized from benzene before use. Potassium chloride was treated with Chelex 100 (Bio-Rad, Richmond, CA) before use. Phosphocreatine (disodium salt) was supplied by Boehringer Mannheim Biochemicals (Indianapolis, IN) and was recrystallized from aqueous ethanol according to the method of Ennor (1957). Creatine was from Aldrich and was recrystallized according to the method of Bloch and Schoenheimer (1939).

Enolase (rabbit muscle) was obtained from Boehringer Mannheim and had a specific activity of 40 units/mg. The enzyme was dialyzed against 50 mM imidazole hydrochloride buffer, pH 7.0, containing $MgCl_2$ (8 mM) and KCl (400 mM). After concentration, the enzyme concentration was 250 mg/mL, or 6.1 mN. Creatine kinase (rabbit muscle) was obtained from Boehringer Mannheim as a stabilized, lyophilized powder and had a specific activity of 550 units/mg. This material was dissolved in 10 mM imidazole hydrochloride buffer, pH 7.0, and then dialyzed against cold, glass-distilled water to remove stabilizers. Subsequent freeze-drying resulted in a fluffy powder that could be stored cold, in the dark, and under argon for at least 6 months without activity loss.

Methods

An Update 1000 rapid-quench apparatus (Update Instruments, Madison, WI) was used in all quench experiments. High-performance liquid chromatography was performed on equipment from Waters (Milford, MA), consisting of two Model 510 pumps, a Model 680 gradient controller, a Model U6K injector, and a Model 441 monochrome detector. Liquid scintillation counting was done on a Beckman LS-1801 instrument. Two types of scintillation cocktail were used, either ScintiVerse II (Fisher Scientific, Medford, MA) for sample volumes up to 16% (v/v) aqueous buffer or Aquasol (New England Nuclear, Billerica, MA) containing 9% (v/v) water

[1] Abbreviations: HPLC, high-performance liquid chromatography; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; Cr, creatine; PCr, phosphocreatine; PGA, 2-phosphoglycerate; PEP, phosphoenolpyruvate; NADH, nicotinamide adenine dinucleotide, reduced form; NAD^+, nicotinamide adenine dinucleotide, oxidized form; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; $NADP^+$, nicotinamide adenine dinucleotide phosphate, oxidized form; AMP, adenosine 5′-monophosphate; Ap_5A, P^1,P^5-bis(5′-adenosyl) pentaphosphate; EDTA, ethylenediaminetetraacetate.

as cosolvent for sample volumes up to 8% (v/v) aqueous buffer. Samples were counted to a precision of better than 1% (2σ). Ultraviolet absorbance was measured by using a Hewlett-Packard 8452A spectrophotometer. Enzymes were concentrated by using Centricon 10 microconcentrators (Amicon Corp., Danvers, MA).

Quench Experiments. A typical experimental arrangement included a multijet mixer (Ballou, 1983) and two syringes (2 mL), one containing quenching agent and the other containing perfluorodecalin. The tubing connecting the perfluorodecalin syringe to the mixer was divided into two segments to facilitate loading of the sample. A volume of perfluorodecalin that exceeded the volume displaced during the rise time of the motor (3.3 ms) separated the sample from the mixer, to ensure that mixing occurred at constant velocity. The quenching agent was 1 M perchloric acid and was mixed with the sample at 3 mL s^{-1} (per syringe) with a total displacement of liquid of 260 μL.

Radioactive substrates (at 10–20 μM) and, where appropriate, cosubstrates and cofactors were added shortly before quenching. After quenching, the acid solution of labeled compounds was separated from perfluorodecalin and denatured enzyme by brief centrifugation at 10000*g*. Radioactivity was localized to the upper, aqueous layer. Nonradioactive substrates and products were added to this solution to aid the subsequent analysis. Samples were stored at 0 °C until analysis, which was always performed within 6 h of quenching. In quench experiments on enolase, the sample contained 50 mM imidazole hydrochloride buffer, pH 7.0, containing $MgCl_2$ (8 mM) and KCl (400 mM). After quenching, 130 μL of the acidic solution was added to a solution (900 μL) containing unlabeled 2-phosphoglycerate (20 μM), phosphoenolpyruvate (20 μM), and EDTA (10 mM) to minimize separation artefacts. In quench experiments on creatine kinase, the sample contained creatine (80 mM) and phosphocreatine (60–130 mM) in 100 mM buffer solution containing $MgCl_2$ (30 mM) and *N*-acetylcysteine (14 mM). Added sodium chloride compensated for the change in ionic strength when the creatine/phosphocreatine ratio was varied. The free magnesium concentration was maintained by changing the concentration of $MgCl_2$ according to

$$M = \frac{M_0}{K_d + M_0} L + M_0 \qquad (1)$$

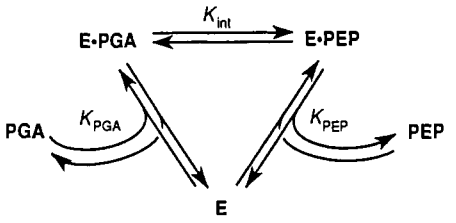
where M is the total concentration of Mg(II), M_0 is the free concentration of Mg(II), L is the total phosphocreatine concentration, and K_d is the dissociation constant of the Mg(II)/phosphocreatine complex (Dawson et al., 1969). After quenching, 130 μL of the acidic solution was added to a solution (900 μL) containing unlabeled ATP and unlabeled ADP (20 μM each) to facilitate detection and EDTA (10 mM) to ensure that any enzyme still present was inactive.

The distribution of radioactivity between substrate pairs was determined by ion-exchange HPLC. When substrate concentrations were quantitated, the radioactivity in the fractions corresponding to each compound was totaled. For analysis of enolase quenches, separation of 2-phosphoglycerate and phosphoenolpyruvate was accomplished on a HR 5/5 Mono Q anion-exchange column (Pharmacia Fine Chemicals, Piscataway, NJ) using a linear gradient (5–500 mM, 15.5 mL plus 15.5 mL) followed by 500 mM triethylammonium formate, pH 3.3 (6 mL), at a flow rate of 2 mL/min. Fractions (0.4 mL) were collected directly into scintillation vials. Both the buffer and the water used as diluent contained EDTA (1 mM). Under these conditions, 2-phosphoglycerate and 3-phosphoglycerate elute together at 24 mL into the gradient, while phosphoenolpyruvate elutes at 34 mL. Both 2-phosphoglycerate and phosphoenolpyruvate are stable under the quench and analysis conditions for the duration of the experiment. The radioactivity that coelutes with these materials could be equilibrated by using enolase and could be converted in greater than 98% yield to a species with a shorter retention time (presumably, lactate) by pyruvate kinase/ADP with lactate dehydrogenase/NADH. More than 80% of the radioactivity introduced into a sample for quenching could be accounted for in the fractions collected for analysis. For analysis of creatine kinase quenches, the labeled substrates [8-^{14}C]ATP and [8-^{14}C]ADP were separated in one of two ways. In the first method, a Du Pont SAX column with Zorbax packing, together with a precolumn of Zipax SAX resin, was eluted with a linear gradient (10 mM–1 M, 7 mL plus 7 mL) of sodium phosphate buffer, pH 3.0. Under these conditions, ADP eluted between 13 and 14 mL into the gradient, and ATP eluted between 16 and 17 mL into the gradient. In the second method, a Pharmacia FPLC Mono Q column was eluted with a linear gradient [1–50% (v/v)] of a mixture of 0.5 M NaH_2PO_4 and 1 M NaCl, pH 4.75, as suggested by Reiss et al. (1984). Under these conditions, ADP eluted between 9.5 and 11 mL into the gradient, and ATP eluted between 12 and 14 mL into the gradient. The major radioactive component eluting from the column was identified as [8-^{14}C]ATP in two ways: by confirming the coelution of the radioactivity with the ultraviolet absorbance and by conversion of the radioactivity into [8-^{14}C]ADP using hexokinase/glucose.

Assays. Enolase concentration was assayed either by following the appearance of phosphoenolpyruvate at 240 nm (Wold & Barker, 1964) or by monitoring the disappearance of NADH at 340 nm when the reaction was coupled to that of phosphoenolpyruvate with pyruvate kinase/ADP and lactate dehydrogenase/NADH (Bergmeyer et al., 1983). The two methods gave identical results. One unit of enzyme activity is defined as that amount which catalyzes the production of 1 μmol of phoshoenolpyruvate/min under these conditions. Extinction coefficients for phosphoenolpyruvate of 1440 M^{-1} cm^{-1} at 240 nm (Wold & Ballou, 1957) and for NADH of 6220 M^{-1} cm^{-1} at 340 nm (Horecker & Kornberg, 1948) were assumed. Creatine kinase was assayed by a modification of the method of Gerhardt (1985), in which ATP formation is coupled to the production of NADH by using hexokinase/glucose and glucose-6-phosphate dehydrogenase/NAD^+. Specific modifications included the omission of adenosine triphosphatase inhibitors such as AMP and Ap_5A and the replacement of the $NADP^+$-dependent glucose-6-phosphate dehydrogenase by the NAD^+-dependent enzyme from *Leuconostoc mesenteroides* as suggested by Wong and Whitesides (1981). The final concentrations of the assay components were 0.1 M imidazole hydrochloride, 2 mM EDTA, 10 mM $MgCl_2$, 2 mM ADP, 20 mM *N*-acetylcysteine, 20 mM glucose, and 2 mM NAD^+. The ADP was kept as a separate solution (stabilized with EDTA) until immediately before use to minimize the level of ATP as a contaminant in the assay mixture. One unit of creatine kinase activity is defined as that amount which catalyzes the production of 1 μmol of ATP/min at 30 °C under these conditions. Protein concentrations were determined from A_{280nm} by using values of $A^{1\%}_{280nm}$ of 0.9 (for enolase) and 0.69 (for creatine kinase).

Assays of phosphocreatine were performed by using essentially the same reactions as for the kinetic assays, above, according to the method of Lamprecht et al. (1984). Assays of

Scheme I: Enolase Reaction[a]



[a] PGA is 2-phosphoglycerate, and PEP is phosphoenolpyruvate. K_{int} is the internal equilibrium constant, and K_{PGA} and K_{PEP} are the dissociation constants for PGA and PEP from the enzyme, respectively.

creatine were performed by coupling the formation of phosphocreatine and ADP, catalyzed by creatine kinase, to the disappearance of NADH by using pyruvate kinase/phosphoenolpyruvate and lactate dehydrogenase/NADH as described in Bernt et al. (1985).

Curve Fitting. Theoretical equations were fitted to experimental data by using an iterative nonlinear least-squares algorithm developed by Brown and Dennis (1972). The algorithm returned the best estimate when the thermodynamic parameters varied by less than 0.1% of their absolute value from iteration to iteration. Initial estimates were obtained by inspection of the experimental data, using the limits of the equations at $[E]_{total} \rightarrow 0$ and at $[E]_{total} \rightarrow \infty$. When equations for two-substrate, two-product enzymes were fitted, the binary dissociation constants of the cosubstrates not being assayed (the substrate–product pair present in excess over enzyme active sites) were assigned values from the literature, as indicated in the figure legends. Variation of these assigned values after minimization of the other parameters did not improve the fit further.

Theory

For a one substrate/one product enzyme such as enolase, consider Scheme I, where K_{PGA} and K_{PEP} represent the dissociation constants of 2-phosphoglycerate and phosphoenolpyruvate from the enzyme, respectively. The ratio of the concentrations of species that will result in phosphoenolpyruvate upon quenching ($[PEP]_{total}$) to those that will result in 2-phosphoglycerate upon quenching ($[PGA]_{total}$) can be written (from Scheme I)

$$\frac{[PEP]_{total}}{[PGA]_{total}} = \frac{[E] + K_{PEP}}{[E] + K_{PGA}} K_{int} \quad (2)$$

where [E] represents the concentration of free (that is, unliganded) enzyme. The concentrations of substrate and product are kept well below that of enzyme, so the free enzyme concentration will be essentially equal to the total enzyme concentration. At low enzyme concentrations ($[E]_{total} \rightarrow 0$), the ratio $[PEP]_{total}/[PGA]_{total}$ that is measured becomes equal to K_{eq}, the overall equilibrium constant.

At high enzyme concentrations, the measured ratio $[PEP]_{total}/[PGA]_{total}$ becomes equal to K_{int}. To confirm that this ratio at high [E] equals K_{int}, the measured ratio must be independent of [E]. If this is true, the data will be fitted by eq 2, and a precise K_{int} can be determined analytically. The midpoint of the curve obtained from plotting $[PEP]_{total}/[PGA]_{total}$ against the total enzyme concentration (which is analogous to the pK_a on an acid–base titration curve) is at $[PEP]_{total}/[PGA]_{total} = (K_{int} + K_{eq})/2$. The total enzyme concentration at this midpoint is equal to the dissociation constant of 2-phosphoglycerate, K_{PGA}. From the curve, therefore, we determine K_{eq} (the ratio of $[PGA]_{total}$ to $[PEP]_{total}$ at low [E]), K_{int} (the ratio of $[PGA]_{total}$ to $[PEP]_{total}$ at high [E]), and K_{PGA} (from the midpoint). The equilibrium relationship then defines K_{PEP}.

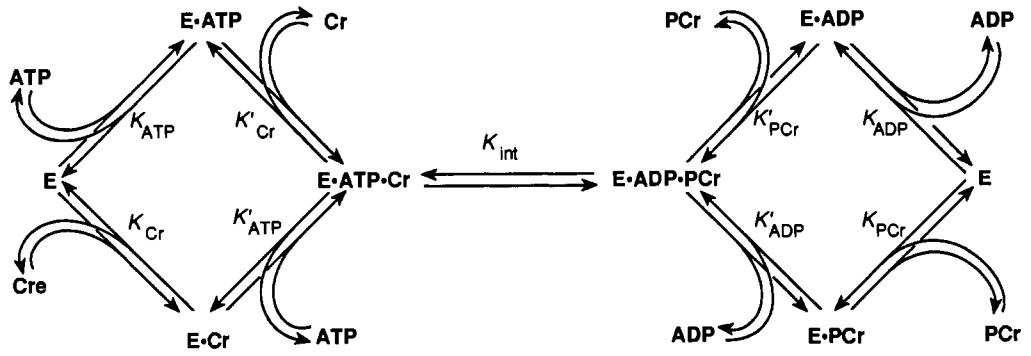
For a two substrate/two product enzyme such as creatine kinase, consider the scheme shown in Scheme II. To measure K_{int}, we choose one of three possible substrate–product pairs (ATP–ADP, Cr–PCr, or ATP–PCr) as the ratio to be measured. By analogy with the one substrate/one product case presented above, the $[ATP]_{total}/[ADP]_{total}$ ratio can be written

$$\frac{[ATP]_{total}}{[ADP]_{total}} = \frac{\left[(K_{ATP} + [E])\frac{K'_{Cr}}{[Cr]} + [E]\right]}{\left[(K_{ADP} + [E])\frac{K'_{PCr}}{[PCr]} + [E]\right]} K_{int} \quad (3)$$

In this case, however, the simplifying assumption used in the case of enolase (that the total enzyme concentration is equal to the free enzyme concentration) is no longer appropriate, because, under such conditions, the labeled substrates would be found largely in binary complexes. To measure K_{int}, the ATP and ADP must be completely bound to the appropriate binary complex, E·Cr or E·PCr, respectively. To eliminate the binary complexes of enzyme with ATP and with ADP, the concentration of the uncomplexed enzyme must be minimized. The concentrations of both free creatine and free phosphocreatine must therefore exceed their binary dissociation constants and must also exceed the highest enzyme concentration that is to be used. Under these conditions, the relationship between the free enzyme concentration [E] and the total enzyme concentration $[E]_{total}$ can be written

$$\frac{[E]_{total}}{[E]} = 1 + \frac{[Cr]}{K_{cr}} + \frac{[PCr]}{K_{PCr}} \quad (4)$$

Scheme II: Creatine Kinase Reaction[a]



[a] Cr represents creatine, and PCr represents phosphocreatine. K_{int} is the internal equilibrium constant, and K and K' are the dissociation constants for substrates and products, as shown.

Substituting eq 4 into eq 3 and simplifying yield

$$\frac{[ATP]_{total}}{[ADP]_{total}} = \left\{\left[[E]_{total} + K'_{ATP}\left(1 + \frac{K_{Cr}[PCr]}{K_{PCr}[Cr]}\right) + \frac{K'_{Cr}}{[Cr]}([E]_{total} + K_{ATP})\right] \Big/ \left[[E]_{total} + K'_{ADP}\left(1 + \frac{K_{PCr}[Cr]}{K_{Cr}[PCr]}\right) + \frac{K'_{PCr}}{[PCr]}([E]_{total} + K_{ADP})\right]\right\} K_{int} \quad (5)$$

If the creatine and phosphocreatine concentrations are kept well above both their respective binary and ternary dissociation constants and above $[E]_{total}$, eq 5 simplifies to

$$\frac{[ATP]_{total}}{[ADP]_{total}} = \left[\frac{[E]_{total} + K'_{ATP}\left(1 + \frac{K_{Cr}[PCr]}{K_{PCr}[Cr]}\right)}{[E]_{total} + K'_{ADP}\left(1 + \frac{K_{PCr}[Cr]}{K_{Cr}[PCr]}\right)}\right] K_{int} \quad (6)$$

As with eq 2, eq 6 simplifies to K_{eq} as $[E]_{total} \rightarrow 0$ and to K_{int} as $[E]_{total} \rightarrow \infty$.

According to eq 6, a plot of $[ATP]_{total}/[ADP]_{total}$ versus $[E]_{total}$ will level off at high $[E]_{total}$, and the value of $[ATP]_{total}/[ADP]_{total}$ extrapolated to infinite $[E]_{total}$ will be independent of the creatine and phosphocreatine concentrations. In addition, if Scheme II is modified to include an E·ADP·Cr abortive ternary complex (Milner-White & Watts, 1971), the limit of $[ATP]_{total}/[ADP]_{total}$ that is reached at infinite $[E]_{total}$ will be related to the K_{int} by

$$\frac{[ATP]_{total}}{[ADP]_{total}} = \frac{K_{int}}{1 + \left(\frac{K''_{ADP}K_{PCr}[Cr]}{K'_{ADP}K_{Cr}[PCr]}\right)} \quad (7)$$

where K''_{ADP} is the dissociation constant of ADP from the abortive ternary complex. This equation predicts that the ratio $[ATP]_{total}/[ADP]_{total}$ observed at high $[E]_{total}$ will depend on the ratio of [Cr] to [PCr]. If the value of $[ATP]_{total}/[ADP]_{total}$ observed at high $[E]_{total}$ is independent of [Cr]/[PCr], abortive ternary complexes are unimportant, and the true K_{int} will be measured.

The success of an equilibrium quench in measuring the value of K_{int} requires that the dissociation rates of substrate and product exceed their interconversion rate on the enzyme. Since the concentrations of the enzymic complexes are derived from the concentrations of the substrate and the product measured after the quench, any interconversion of substrate and product during or after the quench will give a false value for K_{int}. This competition between dissociation and interconversion is illustrated in Figure 2A. If substrate–product interconversion is slow relative to dissociation (that is, if transition state 2 is higher than either transition state 1 or transition state 3, as illustrated by the solid line in Figure 2A), then, provided that all S and P are enzyme bound before the quench, the concentrations of S and P after the quench will equal the starting E·S and E·P concentrations, respectively. On the other hand, if the interconversion reaction is much faster than the ligand "off" rates (as illustrated by the dashed line in Figure 2A), then the concentrations of S and P after the quench will depend only on the partitioning of the equilibrating pool of enzymic complexes (E·S plus E·P) over transition states 1 and 3. The distribution of free substrates and products after the quench will be in accord with the Curtin–Hammett principle (Curtin, 1954).

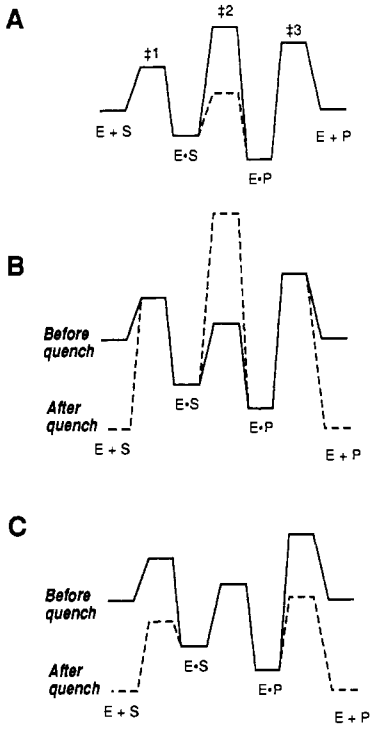


FIGURE 2: Kinetic barrier diagrams that illustrate the consequence of rapid quenching of an enzyme and its substrates at equilibrium: (A) (solid line) where the catalytic interconversion of E·S and E·P is relatively slow, (dashed line) where the catalytic interconversion of E·S and E·P is relatively fast; (B) (solid line) initial equilibrium, (dashed line) after the quench solution has both diluted the sample *and* slowed the interconversion of E·S and E·P; (C) (solid line) initial equilibrium, (dashed line) after the quench solution has both diluted the sample *and* speeded the dissociation of substrate and product from the enzyme. The nature of kinetic barrier diagrams is described in Appendix I of the preceding paper (Burbaum et al., 1989).

Although Figure 2A illustrates the main issues in an equilibrium quench experiment, we cannot assume that, in a quench, the relative heights of kinetic barriers remain the same as those of the normal catalyzed reaction. Thus, quenching agents may change the relative heights of barriers during the course of inactivation, and it is the change in these barriers that can make the choice of quenching agent important. First, however, we discuss the two processes of a quench, mixing (in which the sample and the quenching agent become homogeneous) and inactivation (in which the enzyme loses its catalytic activity).

Several types of mixer have been used for the study of rapid reactions: (i) ball mixers (Berger et al., 1968), (ii) impinging jets (Ballou & Palmer, 1974; Ballou, 1983), and (iii) jets followed by wire mesh screens (Smith, 1973; Hansen, 1977). All three of these types are commercially available or can be constructed easily, and we have compared them experimentally by performing test quenches at high enzyme concentration. We found that the effectiveness of the quench does not depend on the mixer type. The jet mixer was therefore chosen because it is easier to clean and is more durable than the other designs. To determine whether mixing is adequate, the flow velocity of the sample and the quenching agent through the mixer is varied. Because the rate of mixing of two solutions flowing through a pipe depends on the flow velocity and on the critical Reynolds number (which specifies the transition from laminar to turbulent flow), the presumption that a given set of conditions exceeds the critical Reynolds number can be tested directly. In this experiment (a "ram speed control"), the product:substrate ratio after the quench of an equilibrated mixture (at high enzyme concentration) is measured as a

function of the ram speed. If the measured product:substrate ratio is independent of the ram speed, then it may be presumed that mixing is as rapid as the particular mixer design allows.

The consequence of mixing the sample with the quenching agent must be, of course, inactivation of the enzyme, so that every dissociation event (of substrate or product from the enzyme) is irreversible. If the enzyme-catalyzed interconversion of substrate and product is slower than the release of these species from the enzyme into solution, then effective quenching is not difficult, if mixing is fast. On the other hand, if the enzyme is one for which the catalytic step is faster than the substrate and product dissociation steps, then the quenching agent must either slow down the interconversion of E·S and E·P or speed up the dissociation of S and P, or both, in a process that is itself faster than the catalytic interconversion. Effects of the quenching agent on the unliganded enzyme are irrelevant because conversion of E·S to E·P must be at least as fast as the three-step process of release of substrate, binding to a second enzyme, and catalysis by this second enzyme to form product. We focus, therefore, on interactions between the quenching agent and the enzyme/substrate complex.

As an example, let us consider an acid quench. There are several ways in which mixing with acid can provide an effective quench. First, protonation of an essential basic or nucleophilic residue will eliminate catalytic activity. This process is illustrated in Figure 2B, where the free energy barrier to the interconversion is made larger upon acidification. If, however, access of a proton to this essential residue is prevented by substrate, enzyme inactivation will not occur until the substrate or product has dissociated from the active site. In this case, the observed ratio of S to P will be that determined by the relative free energy of transition states 1 and 3, as discussed above. Second, if the enzyme or the substrate has a hydrogen-bond acceptor that is used for binding, then protonation of this site may either accelerate ligand dissociation or slow down catalysis, or both. Schematically, the consequences of accelerating the dissociation of substrates and products are illustrated in Figure 2C, where the free energy barriers to dissociation are made smaller upon quenching. In each of the above cases, protonation is likely to be extremely rapid. Provided that the catalytic residue or substrate group is accessible, protonation will occur with a rate that is limited only by diffusion, so that in 1 M acid the pseudo-first-order rate constant for inactivation will be near 10^{10} s^{-1} (Horne, 1972), compared with typical rate constants for enzyme-catalyzed processes of no more than 10^5 s^{-1}. Moreover, in 1 M acid, protonation will be effectively irreversible, so that any slower process that limits the rate of inactivation will be independent of the concentration of the acid. Whether the inactivation of the enzyme is rapid enough can be addressed directly with an enzyme that has a K_{eq} that is pH dependent. If enzyme inactivation is slow, then the equilibrium constant measured at high enzyme concentration will be the external equilibrium constant at the pH of the quench rather than a value independent of the concentration of the quenching acid.

In conclusion, the choice of acid (or, mutatis mutandis, of base) as the quenching agent combines several possible mechanisms for inactivation. Nevertheless, if the active site of the enzyme in liganded complexes is completely inaccessible and if S or P dissociation is slower than the catalyzed interconversion of E·S and E·P, then mixing the sample solution very rapidly with a large excess of acid may not produce effective inactivation. While the effectiveness of the quench is hard to evaluate, there are some indirect approaches (discussed below) that can make the investigator confident that an effective method has been found.

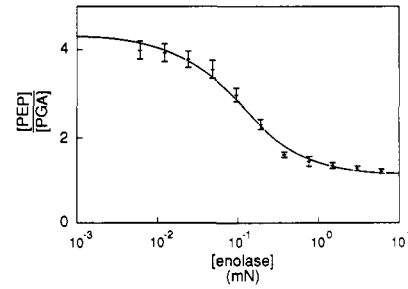


FIGURE 3: Measurement of K_{int} for enolase at pH 7.0, in the presence of Mg(II), 8 mM, and K(I), 400 mM. For further experimental details, see text.

Results and Discussion

One Substrate/One Product Enzymes. Enolase catalyzes the interconversion of phosphoenolpyruvate and 2-phosphoglycerate. The elimination of water from 2-phosphoglycerate is believed to proceed by an E1cB mechanism (that is, the proton at C-2 is removed first to produce an intermediate carbanion) as suggested by isotope-exchange experiments (Dinovo & Boyer, 1971) and by studies using substrate and intermediate analogues (Anderson et al., 1984; Stubbe & Abeles, 1980). The carbanion has not been included explicitly in Scheme I because there is no evidence to suggest that it is a kinetically significant intermediate, and we can expect that it will be thermodynamically less stable than either substrate or product, even when enzyme bound. If the carbanion were to be kinetically significant in the enzymic reaction, this species would either protonate (forming 2-phosphoglycerate) or eliminate (forming phosphoenolpyruvate) on quenching, neither of which would hamper the analysis or the interpretation of the experimental results. Examination of concentrations of 2-phosphoglycerate and of phosphoenolpyruvate in vivo supports the view that this enzyme operates near equilibrium in the cell (Lemieux et al., 1980; Nishiki et al., 1979; Lowry et al., 1964). Consequently, enolase can be classified as a "reversible" enzyme, with a value of θ near 1 (Burbaum et al., 1989), and the theory presented earlier predicts that if enolase is kinetically optimal, it will have a value of K_{int} near unity (Burbaum et al., 1989).

The results of equilibrium quench studies on enolase are shown in Figure 3. These data were obtained under the following conditions: (i) phosphoenolpyruvate of high specific radioactivity was incubated with concentrations of enolase from 5 μM to 5 mM; (ii) the resulting solution was quenched into 1 M perchloric acid; and (iii) the quenched solution was analyzed for 2-phosphoglycerate and phosphoenolpyruvate. The substrate:product ratio appears to follow a titration curve when plotted against log [E], as illustrated. A good correlation is found when eq 2 is fitted to these data, as illustrated by the solid line. The "best" least-squares values[2] for the constants of Scheme I are 0.096 ± 0.01 mM for K_{PGA}, 0.37 ± 0.04 mM for K_{PEP}, and 1.1 ± 0.2 for K_{int}. In addition, the external (overall) equilibrium constant K_{eq} was measured to be 4.3 ± 0.2. These results agree with the work of Brewer and Ellis (1983), who have shown in a ^{31}P NMR study that the value of K_{eq} for the enolase-catalyzed reaction is 4.5 and that the amount of enzyme-bound phosphoenolpyruvate is roughly

[2] The error limits are reported as standard deviations and do not include any error in the determination of the enzyme concentration. Errors in enzyme concentration will affect the values of K_{PGA} and K_{PEP} more seriously than those of K_{int} or K_{eq}.

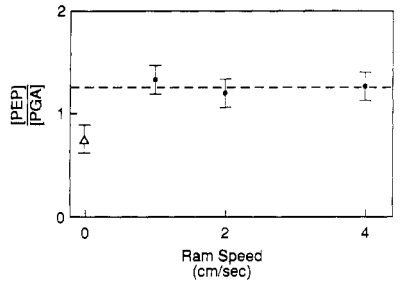


FIGURE 4: Ram speed control for the quenching of the enolase reaction. The enzyme concentration was 1.4 mN. For further details, see Experimental Procedures.

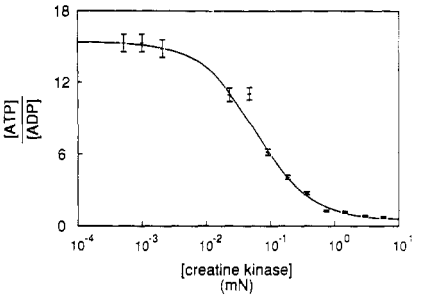


FIGURE 5: Measurement of K_{int} for creatine kinase at pH 8.0, in the presence of Mg(II), 30 mM, [Cr], 80 mM, and [PCr], 120 mM. For further experimental details, see the text.

equal to the amount of enzyme-bound 2-phosphoglycerate.

In Figure 3, the experimental points appear to deviate systematically from the curve fitted to eq 2 as illustrated, although these deviations mostly fall within the error limits of the fit. A more complicated model might fit the data better—such a model would require a higher order dependence of the ratio on enzyme concentration to fit a steeper curve, by analogy to enzymes that show cooperativity. It is very improbable, however, that cooperative binding of the substrate to the enzyme is involved since the concentration of radioactive substrates is small, and it is unlikely that a single enolase dimer will have two such molecules bound simultaneously. More probably, the increasing concentration of enzyme changes either the free [Mg(II)] [enolase has been suggested to bind more than one Mg(II) per subunit; see Brewer and Ellis (1983)] or the solvent characteristics sufficiently to perturb the value of K_{eq}, K_{PGA}, or K_{PEP}. For present purposes, however, the apparent deviation is of no importance.

As discussed above, the value of K_{int} obtained from the fitting of eq 2 to the experimental data could be inaccurate if the mixing were inadequately rapid. We can change the efficiency of mixing by varying the velocity of the sample and acid as they pass through the mixer. If mixing is fast enough, then varying the flow rate through the mixer will have no effect on the derived value of K_{int}. As illustrated in Figure 4, changes in flow rate have no effect on the substrate:product ratio that is observed at high concentrations of enzyme. There are two cases where the independence of results on the flow rate might be observed: if mixing is fast enough for K_{int} to be observed or if mixing is never fast enough and a value of K_{eq} is seen. To test which situation is relevant, the enzyme was quenched *very* slowly, and a smaller ratio was observed (the open triangle in Figure 4). This smaller value is, indeed, that expected for the external equilibrium constant at low pH (Wold & Ballou, 1957). Mixing is evidently fast enough.

Up to this point, we have skirted the issue of the fate of the putative carbanion intermediate in the enolase reaction. As discussed above, this intermediate is not likely to play a significant thermodynamic role, yet if it were kinetically significant and were to dissociate from the enzyme on quenching, protonation would result in racemic phosphoglycerate. However, all of the radioactivity after the quench can be converted enzymatically into lactate, so the 2-phosphoglycerate after quenching is all of the natural D configuration. Either the equilibrium concentration of enzyme-bound carbanion is small or the carbanion is protonated before its (irreversible) release from the enzyme.

Two Substrate/Two Product Enzymes. Creatine kinase is abundant in muscle tissue and is responsible for catalyzing the reversible phosphorylation of creatine. On the basis of the early studies of Meyerhof and Lohmann (1928) and the observation that phosphocreatine (but not ATP) is depleted in muscles during contraction, the role of phosphocreatine as an intracellular energy store (and of creatine kinase as its broker) was established shortly thereafter (Lohmann, 1934; Lundgaard, 1938). Phosphorylating equivalents produced at rest are stored as phosphocreatine, and, during muscle contraction, this phosphocreatine reserve is used to maintain intracellular ATP levels [see Kushmerick (1983)]. Although this simple view has been questioned and it has been proposed that two creatine kinase isozymes are responsible for the transport of phosphorylating equivalents between the mitochondrion and the myofibril (Bessman, 1972; Bessman & Geiger, 1981), recent NMR studies of whole muscle show that the rate of conversion of phosphocreatine to ATP is very close to the rate of conversion of ATP to phosphocreatine (Ugurbil et al., 1986; Degani et al., 1985). Creatine kinase therefore operates close to equilibrium in vivo, and in terms of the theory developed in the previous paper (Burbaum et al., 1989), $\theta = 1$, and K_{int} is predicted to be unity.

The determination of K_{int} for creatine kinase presents a somewhat more complicated problem than that for enolase, but this very complexity allows more exacting controls to be performed. Thus, to demonstrate that our measurement of K_{int} is accurate, we can vary not only the concentration of the enzyme but also the concentration of the cosubstrates that are not involved in the measurement of K_{int}. In the determination of K_{int} for creatine kinase, we chose ATP and ADP as the conjugate radioactive markers, so eq 3–7 (rather than the analogous equations for other conjugate pairs) describe the experiments performed. We preferred the two nucleotides as the marker substrates both because the rapid acid-catalyzed dephosphorylation of phosphocreatine would have ruled out an acid quench and because it seemed likely that the nucleotide pair would bind more tightly to the enzyme. Furthermore, the separation of ATP and ADP is straightforward, and the method of analysis developed for creatine kinase can be extended to other kinases.

The results shown in Figure 5 were obtained under the following conditions: (i) the creatine kinase solution, and the solution with which it was diluted, contained sufficient creatine and phosphocreatine (each enough to saturate the enzyme) to ensure that all the enzyme complexes containing ATP and ADP were ternary; (ii) ATP of high specific radioactivity was incubated with concentrations of creatine kinase from about 0.5 μM to 5 mM; (iii) the resulting mixture was quenched into 1 M perchloric acid; and (iv) the quenched mixture was analyzed for ATP and ADP. The simplifying assumptions that precede eq 6 guided the design of the experiment. Since the term that depends on the concentrations of substrates in either the numerator or the denominator of eq 6 can overwhelm the corresponding term dependent on the concentration of enzyme, the choice of the [PCr]/[Cr] ratio must ensure that neither binary complex dominates. If the concentrations of creatine

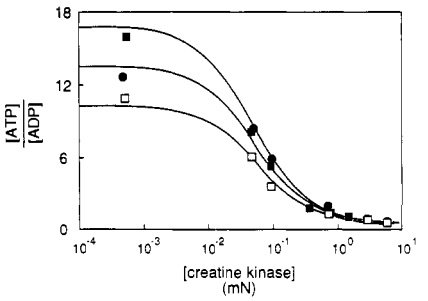


FIGURE 6: Demonstration that variation of the ratio of phosphocreatine (PCr) to creatine (Cr) has no effect on the value of K_{int} for the creatine kinase reaction. The experimental conditions were as for the experiments shown in Figure 5, except for the following: (■) [PCr] = 130 mM, [Cr] = 80 mM (so [PCr]/[Cr] = 1.6); (●) [PCr] = 90 mM, [Cr] = 80 mM (so [PCr]/[Cr] = 1.1); (□) [PCr] = 60 mM, [Cr] = 80 mM (so [PCr]/[Cr] = 0.8).

Table I: Effect of the Variation of the Creatine/Phosphocreatine Ratio on the Thermodynamic Parameters of Creatine Kinase at pH 8.0

[PCr]/[Cr]	K'_{ADP} (mM)	K'_{ATP} (mM)	K_{int}
1.63	0.043 ± 0.013[a]	0.55 ± 0.25	0.42 ± 0.12
1.47	0.028 ± 0.012	0.36 ± 0.19	0.49 ± 0.14
1.14	0.078 ± 0.051	1.15 ± 1.25	0.38 ± 0.25
0.82	0.036 ± 0.028	0.56 ± 0.54	0.45 ± 0.21
consensus[b]	0.040 ± 0.009	0.48 ± 0.14	0.50 ± 0.08

[a] Errors are reported as standard deviations. [b] Obtained by fitting all four sets of experimental data to a single set of equilibrium constants.

and phosphocreatine are chosen to be both saturating and in rough proportion to the binary dissociation constants (so that $[PCr]/[Cr] \approx K_{PCr}/K_{Cr}$), then the total amount of enzyme, $[E]_{total}$, needed to reach K_{int} is minimized (provided that $K'_{ATP} \approx K'_{ADP}$).

The experimental conditions for the initial study [pH 8.0 and [Mg(II)] = 1.7 mM] were chosen because the binary dissociation constants for creatine and phosphocreatine have been established under these conditions (Morrison & James, 1965). On the basis of these dissociation constants, ratios of [PCr]/[Cr] between about 0.1 and 2.5 were indicated. Accordingly, a [PCr]/[Cr] ratio of 1.6 was used, and the data points were fitted to eq 6. The constants that result predict the curve of Figure 5. Because each curve can be described by three variables at most (the two end points and the midpoint), only three variables (K'_{ADP}, K'_{ATP}, and K_{int}) were allowed to vary in the fitting procedure. Literature values were assumed for the binary dissociation constants K_{PCr} and K_{Cr}, and the fit of the curve to the experimental points in Figure 5 is gratifying.

To prove that an accurate value of K_{int} is determined from Figure 5, the [PCr]/[Cr] ratio was varied from 1.6 to 0.8, and the data and the curves that result are plotted in Figure 6. These results show that while the [ATP]/[ADP] ratio at low enzyme concentration varies as predicted from K_{eq}, the [ATP]/[ADP] ratio at high enzyme concentration (that is, K_{int}) is constant. This comparison demonstrates that the two radioactive nucleotides ATP and ADP are predominantly bound in ternary complexes with the appropriate cosubstrate (rather than in either of the nonproductive ternary complexes or in binary complexes) and that the value of the ATP/ADP ratio at high enzyme concentrations indeed measures K_{int}. In the set of experiments illustrated in Figures 5 and 6, the concentration of creatine was fixed and the concentration of phosphocreatine was varied. Both the free magnesium concentration and the ionic strength were maintained by adding KCl and extra Mg(II) according to the literature dissociation

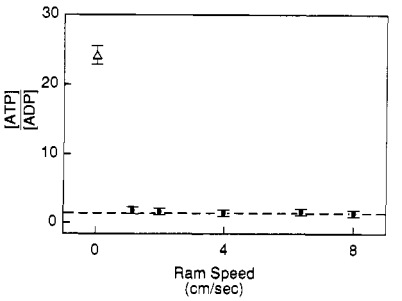


FIGURE 7: Ram speed control for the quenching of the creatine kinase reaction at pH 8.0. The enzyme concentration was 1.5 mN; [PCr] = 120 mM, and [Cr] = 80 mM. For further details, see Experimental Procedures.

Table II: Effect of the Variation of pH on the Thermodynamic Parameters of Creatine Kinase

pH	K'_{ADP} (mM)	K'_{ATP} (mM)	K_{int}
6.8	0.04 ± 0.03	0.3 ± 0.2	5.8 ± 1.9
7.2	0.08 ± 0.04	0.7 ± 0.3	4.3 ± 0.9
7.6	0.02 ± 0.01	0.2 ± 0.1	2.5 ± 0.9
8.0	0.04 ± 0.01	0.6 ± 0.3	0.4 ± 0.1
8.4	0.01 ± 0.003	0.3 ± 0.1	0.3 ± 0.1

constant of Mg·PCr of 50 mM (Smith & Alberty, 1956). The constants that result from the fitting of eq 6 to each set of experiments are tabulated in Table I. Note that the errors in the apparent dissociation constants K'_{ATP} and K'_{ADP} appear larger than the errors in K_{int}. This may be due to variations in the concentration of enzyme between curves: the nucleotide dissociation constants are related to the concentration of enzyme, whereas K_{int} is independent of enzyme concentration. In an analogous experiment (data not shown), variation of the creatine concentration was shown to have no effect on the value of K_{int}.

Because the value of K_{int} extrapolated from each of the four curves is independent of [PCr]/[Cr] (rather than being either proportional to or inversely proportional to this ratio), the assumptions used in deriving eq 6 are evidently valid. To improve the statistical significance of the results, all four sets of data were fitted to a single set of constants, shown as the "consensus" entry in Table I.

The value of K_{int} that we have obtained might be inaccurate due to consistently slow mixing, as discussed above. Since the rate of inactivation is likely to depend on the lability of the particular enzyme, a ram speed control experiment is required in each case. This control experiment for creatine kinase is illustrated in Figure 7, which shows that the rate of mixing is fast enough in this case also. Further, since the value of K_{eq} for the creatine kinase reaction rises at low pH (Lawson & Veech, 1979), if the enzyme were inactivated too slowly in the acid quench experiment, the ratio of [ATP]/[ADP] would increase at high enzyme levels, as is illustrated by the open triangle (for very slow quenching) in Figure 7. Instead, the ratio of [ATP]/[ADP] falls as the enzyme concentration increases (see Figures 5 and 6), further validating the quenching effectiveness.

The equilibrium constant of the creatine kinase reaction is pH dependent, and to understand further the internal thermodynamics of the creatine kinase reaction, quenching experiments were repeated at several pH values. The value of K_{eq} for the creatine kinase reaction varies with pH as expected (Lawson & Veech, 1979). When the experiments described above were repeated at several pH values between 6.8 and 8.4, the curves shown in Figure 8 and the values of K_{int} listed in Table II were obtained. Somewhat surprisingly, the value of K_{int} varies significantly with pH. In principle, the binding

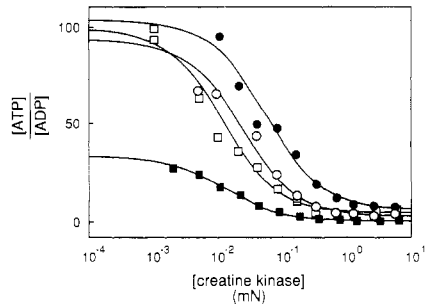


FIGURE 8: Observed ratios of ATP and ADP after quenching equilibrium mixtures of the complete creatine kinase reaction mixture, as a function of the total creatine kinase concentration. The pH values and the molar ratios of phosphocreatine to creatine (in parentheses) were (●) pH 6.8 (1.5), (□) pH 7.2 (1.6), (○) pH 7.6 (1.5), and (■) pH 8.4 (1.5).

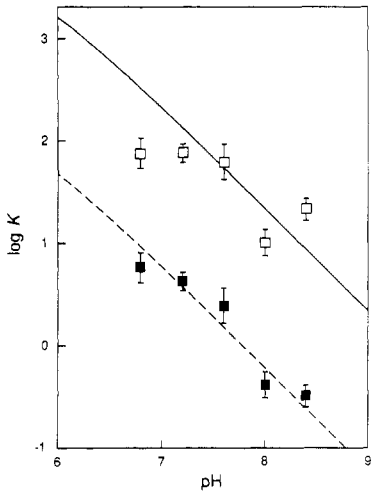


FIGURE 9: Plot of log K_{int} (■) and log K_{eq} (□) for the creatine kinase reaction as a function of pH. The solid line is that predicted from the analysis of the pH dependence of K_{eq} for the creatine kinase reaction (Lawson & Veech, 1979). The dashed line is drawn parallel to the solid line through the solid symbols.

constants of the substrates and products could have changed to allow the value of K_{int} to remain constant. Yet, as can be seen from Figure 9, the value of K_{int} varies with pH in the same way as the overall equilibrium constant, K_{eq}.[3] This correlation between K_{int} and K_{eq} shows that the proton involved in the external equilibrium (formally, the imino proton that is lost upon phosphorylation of creatine) is lost to solution upon interconversion of the ternary complexes (that is, the process is described by E·ADP·PCr + H^+ ⇄ E·ATP·Cr). In support of this result, the kinetic parameters of creatine kinase vary with pH in a manner that is consistent with a freely exchanging proton in the active, ternary complex (Cook et al., 1981). All the experiments thus indicate that the ternary complexes are in protonic equilibrium with the medium. Finally, we may note that an earlier determination of K_{int} for creatine kinase by Leyh et al. (1985) of 0.75 at pH 8.0, which also involved the quenching of equilibrium mixtures of enzyme and substrates into acidic solution, is in close agreement with the corresponding value reported here (Figure 9).

[3] Figure 9 shows the variation of the values of K_{eq} both in our experiments and in the more detailed experiments of Lawson and Veech (1979). The values of K_{eq} from our experiments (from Table II) are plotted as open squares, and the values from the earlier studies are represented by the solid line. The deviations of our data from the line predicted by the earlier studies can be ascribed to the random variations inherent in dealing with necessarily small samples. The deviations of the K_{int} values from the theoretical line appear to follow the deviations of the K_{eq} values from their expected values, suggesting that the errors in determining K_{eq} are mirrored by the errors in determining K_{int}.

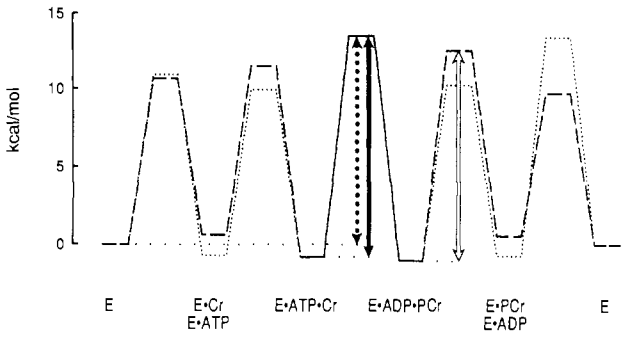


FIGURE 10: Kinetic barrier diagram for the reaction catalyzed by creatine kinase at pH 8, when substrates and products are at their physiological concentrations. The solid arrow represents the barrier to interconversion of the ternary complexes, which limits the rate of enzyme turnover under in vivo conditions. The dotted arrow and the open arrow represent the barriers that determine the uniform-binding criterion (see the text). The dashed line profile illustrates the situation when creatine (Cr) and phosphocreatine (PCr) bind first to the free enzyme (E); the dotted line profile illustrates the situation when ATP and ADP bind first to the free enzyme. The following parameters were used: $k_D = 2 \times 10^7$ M^{-1} s^{-1} (Hammes & Hurst, 1969); K_{eq} = 0.083 (Lawson & Veech, 1979); k_{cat} (ATP to ADP) = 305 s^{-1} and k_{cat} (ADP to ATP) = 160 s^{-1} (Cook et al., 1981). The substrate and product concentrations are [Cr] = 6 mM, [ATP] = 4 mM, [PCr] = 30 mM, and [ADP] = 67 μM (Kushmerick, 1983), where [ADP] is derived from the equilibrium relationship. The total enzyme concentration in the cell is taken as 0.2 mM (Kushmerick, 1983). A computer simulation of the equilibration of the enzyme with its substrates and products allowed the determination of the concentration of each complex on the reaction path (Burbaum, 1988).

To illustrate the results we have obtained and to demonstrate the correspondence of these results with the theory developed in the previous paper (Burbaum et al., 1989), we summarize the kinetic and thermodynamic constants measured here and elsewhere in the kinetic barrier diagram shown in Figure 10. Creatine kinase provides an unusual opportunity to test our proposals, because the concentrations of the substrates and products of the creatine kinase reaction are among the most certain in physiology. Inspection of Figure 10 reveals that the diagram is in accord with our theory (Burbaum et al., 1989). The kinetic barrier that limits the rate at which the enzyme interconverts its substrates and its products (that is, the barrier that corresponds to the dominant term in the efficiency equation) is the conversion of one ternary complex into the other, as illustrated in Figure 10 by the solid arrow. The *uniform-binding* criterion is satisfied: the substrates are bound neither too tightly (which would result in the open arrow becoming dominant) nor too loosely (which would result in the dotted arrow becoming dominant). Further, since the chemical interconversion step remains important for the turnover of the enzyme, the forces of *differential binding* remain important, optimization of which results in a K_{int} close to unity, as predicted.

The General Case. In the preceding paper (Burbaum et al., 1989), we have presented a view of the energetic characteristics of enzymes that have become optimal with respect to catalytic efficiency in a particular metabolic niche. The defining feature of such a niche is the ratio of the steady-state levels of substrate (which is being provided by the action of the upstream enzymes in the metabolic sequence) and product (which is being swept on by the action of the downstream enzymes) to the ratio of these concentrations at equilibrium. While most enzymes evidently maintain their substrates and products close to equilibrium in vivo (so that these systems operate under essentially reversible conditions), there are other enzymes, no-

Table III: Values of the Equilibrium Constant for Enzyme-Bound Species, K_{int}

enzyme	K_{int}	K_{eq}	method[a]	θ	ref
$\theta = 1$					
adenylate kinase	0.6	2.6	N	1	*k*
arginine kinase[b]	1.3	2.0	K	1	*l*
arginine kinase[b]	1.3	0.1	N	1	*m*
creatine kinase[b]	0.9	0.02	K	1	*n*
creatine kinase[b]	~1	0.08	N	1	*o*
creatine kinase[b]	2.5	0.08	EQ	1	*p*
enolase[c]	~1	5.0	N	1	*q*
enolase[c]	1.1	4.3	EQ	1	*p*
glutamine synthase	1.9	$\sim 10^3$	K	1	*r*
lactate dehydrogenase[b]	4	3×10^4	K	1	*s*
phosphoglucomutase	2.5	17	EQ	1	*t*
phosphoglycerate kinase	0.8	$\sim 10^{-4}$	N	1	*u*
θ unknown					
carbamoyl phosphate synthase[d]	11	$\sim 10^8$	K	–	*v*
EPSP synthase	17	180	K, SQ	–	*w*
isoleucyl-tRNA synthetase[e]	0.2	$\sim 10^{-7}$	K	–	*x*
methionyl-tRNA synthetase[e]	1.7	$\sim 10^{-7}$	N	–	*y*
myosin ATPase[d]	9	7×10^5	K	–	*z*
S-adenosylmethionine synthetase[c,f]	2	2×10^5	SQ	–	*aa*
tyrosyl-tRNA synthetase[e]	2.3	$\sim 10^{-7}$	K	–	*bb*
$\theta < 1$					
dihydrofolate reductase	1600	4×10^3	K	10^{-2}[g]	*cc*
fructose 1,6-bisphosphatase[c]	>100	3×10^2	K, SQ	10^{-4}[h]	*dd*
hexokinase	*i*	$\sim 10^3$	SQ	10^{-5}[i]	*ee*
pyruvate kinase	*j*	3×10^3	EQ	10^{-2}[j]	*ff*

[a] N, NMR determination; K, kinetic determination; SQ, steady-state quench; EQ, equilibrium quench. [b] These reactions are presumed to be at equilibrium in vivo ($\theta = 1$) because their products are not used elsewhere in metabolism. [c] For enzymes that involve water as a substrate, unit activity is assumed for H_2O. [d] For HCO_3^--induced ATPase. K_{eq} is calculated from the free energies of hydrolysis of carboxyphosphate given in Sauers et al. (1975) and of ATP given in Jencks (1970). [e] Values are for the first half-reaction (adenylylation of the cognate amino acid). [f] For $PPP_i \rightarrow PP_i + P_i$ reaction only. [g] θ for DHFR was determined from the following substrate concentrations and ratios: [THF]/[DHF] = 3–50 (Doig et al., 1984); [NADPH] = 0.12 mM, [NADPH] = 0.37 mM (Williamson et al., 1969). [h] θ for fructose bisphosphatase was determined from the following substrate concentrations found in rat liver: [fructose 1,6-bisphosphate], 3 μM; [fructose 6-phosphate], 9 μM (Williamson et al., 1969); [P_i] = 4 mM (Veech et al., 1972). [i] The determination of K_{int} for hexokinase is discussed in the text. θ for hexokinase was determined from the following substrate concentrations found in rat heart: [glucose] = 0.43 mM; [ATP] = 4.7 mM; [glucose 6-phosphate] = 59 μM; [ADP] = 0.54 mM (Williamson, 1965). [j] In this measurement, stoichiometric amounts of enzyme, ATP, and pyruvate were used. Because K_{eq} for the pyruvate kinase reaction is large, however, these conditions do not produce enough phosphoenolpyruvate to saturate the enzyme, and the requirements of eq 6 are not satisfied. Further, at these concentrations of enzyme and pyruvate, the enzyme catalyzes the decomposition of pyruvate (Burbaum, 1988) in addition to the loss of pyruvate by the Mg(II)-catalyzed aldol condensation (Gallo & Sable, 1973). When the equilibrium quench experiment is repeated under conditions where the requirements of eq 6 are satisfied throughout the course of the incubation, no titration was observed. The K_{int} for pyruvate kinase is, therefore, controversial. θ for pyruvate kinase was determined from the following substrate concentrations found in rat heart: [pyruvate] = 16 μM; [ATP] = 4.6 mM; [PEP] = 6 μM; [ADP] = 0.86 mM (Williamson, 1966). [k] At pH 7.0; Nageswara Rao et al., 1978a. [l] At pH 8.6; Barman et al., 1978. [m] At pH 7.3; Nageswara Rao et al., 1976. [n] At pH 8.6; Travers et al., 1979. [o] At pH 8.0; Nageswara Rao et al., 1981. [p] This work, see the text. [q] At pH 7.9; Brewer & Ellis, 1983. [r] At pH 7.5; Meek et al., 1982. [s] At pH 7.0; Gutfreund & Trentham, 1975. [t] At pH 7.5; Ray & Long, 1976b. [u] At pH 8.0; Nageswara Rao et al., 1978b. [v] At pH 7.5; Raushel & Villafranca, 1979. [w] At pH 7.0; Anderson et al., 1988. [x] At pH 8.0; Holler & Calvin, 1972. [y] At pH 7.6; Fayat et al., 1980. [z] Trentham et al., 1976. [aa] Markham, 1987; Mudd & Mann, 1963. [bb] At pH 7.8; Wells & Fersht, 1986. [cc] At pH 7.5; Fierke et al., 1987. [dd] At pH 7.6; Benkovic & deMaine, 1982; Rahil et al., 1982; Lawson & Veech, 1979. [ee] At pH 7.5; Wilkinson & Rose, 1979. [ff] At pH 8.0; Stackhouse et al., 1985.

tably those at metabolic control points, that cannot (and these systems operate under more or less irreversible conditions). We have defined the parameter θ (which equals $[P]/[S]K_{eq}$) to specify how closely an enzyme achieves reversible conditions in vivo. Systems that operate reversibly have values of θ near unity, and systems that operate irreversibly have values of θ less than unity. In terms of the theory presented, *any enzyme that operates reversibly* (for which $\theta \rightarrow 1$) *is predicted to have a value of K_{int} of unity*, whatever the value of K_{eq} and whatever the value of k_{cat} for the catalyzed reaction. *Any enzyme that operates irreversibly* (for which $\theta < 1$) *is predicted to have a value of K_{int} that falls between 1 and $1/\theta$.*

How do real systems behave? In Table III, we have listed all the experimental values of K_{int} known to us. It is evident that *all those systems that operate near equilibrium in vivo have values of K_{int} that are strikingly near unity.* These data support the view put forward earlier (Albery & Knowles, 1976) that kinetically optimal enzymes operating under reversible conditions will match the free energies of their internal states. Table III also lists four enzymes for which $\theta < 1$ (that is, enzymes that do not manage to equilibrate their substrates and products in vivo). The experimental values of K_{int} are unambiguous in two of these cases, and we see values of K_{int} that differ from unity in the direction predicted by the theory (Burbaum et al., 1989). For a third enzyme, hexokinase, it has been shown (Wilkinson & Rose, 1979) that the concentrations of the two ternary complexes under conditions that approximate the in vivo steady state are also in accord with the theory.[4] In summary, the results collected in Table III are in excellent agreement with the ideas presented in this and the preceding paper (Burbaum et al., 1989) and provide encouraging support for the views on the development of catalytic efficiency developed therein.

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[4] It was also argued (Wilkinson & Rose, 1979) that the ternary complexes are at equilibrium, but, as discussed earlier (Burbaum et al., 1989), this is less critical than that the barriers to the turnover of enzyme/substrate and enzyme/product complexes be matched.

Registry No. ADP, 58-64-0; ATP, 56-65-5; PGA, 2553-59-5; PEP, 138-08-9; Cr, 57-00-1; PCr, 67-07-2; Cr kinase, 9001-15-4; enolase, 9014-08-8.

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Differences in the Effects of Phorbol Esters and Diacylglycerols on Protein Kinase C†

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ABSTRACT: The binding of protein kinase C (PKC) to membranes and appearance of kinase activity are separable events. Binding is a two-step process consisting of a reversible calcium-dependent interaction followed by an irreversible interaction that can only be dissociated by detergents. The irreversibly bound PKC is constitutively active, and the second step of binding may be a major mechanism of PKC activation [Bazzi & Nelsestuen (1988) *Biochemistry 27*, 7589]. This study examined the activity of other forms of membrane-bound PKC and compared the effects of phorbol esters and diacylglycerols. Like the membrane-binding event, activation of PKC was a two-stage process. Diacylglycerols (DAG) participated in forming an active PKC which was reversibly bound to the membrane. In this case, both activity and membrane binding were terminated by addition of calcium chelators. DAG functioned poorly in generating the constitutively active, irreversible PKC–membrane complex. These propreties differed markedly from phorbol esters which activated PKC in a reversible complex but also promoted constitutive PKC activation by forming the irreversible PKC–membrane complex. The concentration of phorbol esters needed to generate the irreversible PKC–membrane complex was slightly higher than the concentration needed to activate PKC. In addition, high concentrations of phorbol esters (≥50 nM) activated PKC and induced irreversible PKC–membrane binding in the absence of calcium. Despite these striking differences, DAG prevented binding of phorbol esters to high-affinity sites on the PKC–membrane complex. Taken together, the results may suggest that a low-affinity interaction between PKC, phorbol esters, and/or the membrane component was responsible for the irreversible membrane-binding event that produced the constitutively active kinase. These different behaviors of DAG and phorbol esters may be consistent with their different and complex effects in whole cells and tissues.

Phorbol esters are potent tumor-promoting agents that elicit a variety of biological responses (Blumberg et al., 1984; Ashendel, 1985; Blumberg, 1988) which may stem from their effect on potein kinase C (PKC).[1] The activity of this enzyme is sensitive to calcium and diacylglycerol and is believed to be involved in signal transduction by the phosphatidylinositol cycle (Nishizuka, 1986a,b; Kikkawa & Nishizuka, 1986). Phorbol esters and diacylglycerol (DAG) are often thought to exert their function via the common mechanism of PKC activation. The more pronounced effects of phorbol esters may stem from the rate of metabolism. DAG is rapidly metabolized and will activate PKC in a transitory fashion while phorbol esters are degraded very slowly and can activate PKC over a longer time (Nishizuka, 1986a).

Phorbol esters alter the cellular distribution of PKC by producing a stable membrane-associated form that can only be solubilized with detergents (Kraft et al., 1982; Kraft &

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[1] Abbreviations: BSA, bovine serum albumin; DAG, diacylglycerol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PC, phosphatidylcholine; PDBu, phorbol 12,13-dibutyrate; PKC, Ca^{2+}- and phospholipid-dependent protein kinase; PMA, phorbol 12-myristate 13-acetate; PS, phosphatidylserine.

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