Effects of Primary Sequence Differences on the Global Structure and Function of an Enzyme: A Study of Pyruvate Kinase Isozymes†

Thomas G. Consler,‡ Suzanne H. Woodard, and James C. Lee*

E. A. Doisy Department of Biochemistry, St. Louis University School of Medicine, St. Louis, Missouri 63104

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ABSTRACT: Pyruvate kinase is an important glycolytic enzyme which is expressed differentially as four distinct isozymes whose catalytic activity is regulated in a tissue-specific manner. The kidney isozyme is known to exhibit sigmoidal kinetics, whereas the muscle isozyme exhibits hyperbolic kinetic properties. By integration of the crystallographic [Stuart, D. I., Levine, M., Muirhead, H., & Stammers, D. K. (1979) *J. Mol. Biol. 134*, 109–142] and primary sequence data [Noguchi, T., Inoue, H., & Tanaka, T. (1986) *J. Biol. Chem. 261*, 13807], it was shown that the primary sequence for the Cα1 and Cα2 regions may constitute the allosteric switching site. To provide insights into the effects of the localized sequence change on the global structural and functional behavior of the enzyme, kinetic studies under a wide spectrum of conditions were conducted for both the muscle and kidney isozymes. These conditions include measurements of enzyme activity as a function of substrate concentrations with different concentrations of allosteric inhibitors or activators. These results showed that both isozymes exhibit the same regulatory properties although quantitatively the distribution of active and inactive forms and the various dissociation constants which govern the binding of substrate and allosteric effectors with the enzyme are different. For such a majority of equilibrium constants to be altered, the localized primary sequence change must confer global pertubations which are manifested as differences in the various equilibrium constants. Structural information about these two isozymes was provided by phase-modulation measurement of the fluorescence lifetime of tryptophan residues under a variety of experimental conditions. The results are consistent with the kinetic data in indicating that the kidney isozyme exists mostly in the inactive form at pH 7.5, 23 °C, while the muscle isozyme is present almost exclusively in the active form. Results from collisional quenching and proteolytic digestion experiments imply that the kidney isozyme either assumes a less compact structure or undergoes more dynamic motions than the muscle isozyme. Hence, both structural and kinetic informations infer that the localized sequence change exert long-range effects on the global behavior of these enzymes.

The coordinated synthesis of tissue-specific isozymes is a common mechanism for the precise regulation of important metabolic reactions and pathways based on the specific environmental demands of the cell. A mechanism that leads to the production of protein isoforms is that of alternative splicing of messenger RNA (Breitbart et al., 1987). Often the differences among these alternatively spliced mRNAs (and therefore the protein sequence they encode) comprise an exchange of only a small, localized region of their sequence. These regional changes in amino acid sequence generally can be translated into subtle variations in specificity and regulatory behaviors exhibited by these particular isozymes. The specific mechanism(s) that link(s) structural to functional changes are generally not well defined. A system that is particularly well suited for study to provide this type of information is that of pyruvate kinase.

Pyruvate kinase (PK)[1] is an important glycolytic enzyme which is expressed differentially in a tissue-specific manner. There are four distinct isozymes, and each is endogenous primarily to a certain tissue type: liver (L), erythrocyte (R), skeletal muscle (M_1), and kidney (M_2). The reaction catalyzed by PK is also regulated in a tissue-specific manner. The mode of regulation for each isozyme reflects the metabolic requirements of the tissue in which it is preferentially expressed. Pyruvate kinase isozymes found in gluconeogenic tissues have been described as being under allosteric control. In contrast, the muscle isozyme is *not* generally regarded as exhibiting allosteric properties (Hall & Cottam, 1978). In spite of such diverse regulatory behavior, the primary mechansim of catalysis is apparently preserved among isozymes. Since the observed enzymic activity is the consequence of a series of reactions (e.g., substrate and effector binding, protein isomerization, etc.), the change in the primary sequence may affect a specific equilibrium, e.g., isomerization, which ultimately leads to a change in the regulatory behavior, or the sequence change may exert a global effect on the enzyme, leading to changes in most if not all of the equilibria characterizing the enzyme reaction. At present there is no information on the pyruvate kinase system that would enable us to differentiate between these two possible mechanisms.

In a series of reports, Oberfelder et al. (1984a,b) investigated the mechanism of regulation of muscle PK by kinetic, equilibrium, and structural measurements. The simplest model which seems to rationalize the experimental data is a concerted, allosteric model analogous to the two-state model of Monod–Wyman–Changeux (Monod et al., 1965). Recently, Consler et al. (1988) reported that the conformational transition underlying the regulation of PK activity involves a contraction

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* To whom correspondence should be addressed.

‡ Present address: Institute of Molecular Biology, University of California, Los Angeles, CA 90024-1570.

[1] Abbreviations: PK, pyruvate kinase; M_1-PK and M_2-PK, muscle and kidney isozymes, respectively; FBP, fructose 1,6-bisphosphate; PEP, phosphoenolpyruvate; Phe, L-phenylalanine; TKM buffer, 50 mM Tris, 72 mM KCl, and 7.2 mM $MgSO_4$ at pH 7.5.

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and expansion of the global structure of the enzyme regulated by the presence of metabolites. This change in the hydrodynamic properties was characterized by a rotation of one domain (domain B) relative to the remainder of the subunit. Phosphoenolpyruvate, Mg^{2+}, and K^+, the cations required for catalysis, are the ligands that facilitate the domains to move closer to each other, "priming" the enzyme for catalytic activity. Phe and high pH, on the other hand, cause this interdomain region to open up, rendering the enzyme inactive. However, these domain movements only affect the local region around the active site of each subunit. On the basis of X-ray crystallographic data (Stammers & Muirhead, 1977; Stuart et al., 1979), the interdomain region which is involved in cleft closure does not participate directly in intersubunit contacts; thus, it is highly unlikely that this structural region is responsible for the intersubunit contact that leads to the cooperative nature of the structural change. The cooperativity has been indicated by the observation that the binding of one Phe molecule per PK tetramer can induce approximately 80% of the observed hydrodynamic structural changes (Oberfelder et al., 1984a). Hence, additional conformational changes must accompany the domain movement, and these must involve intersubunit contact sites so that conformational communication exists among the four subunits.

Among the isozymes, there is a near-identical preservation of active site residues. There is, however, a distinct difference observed in the amino acid sequences of the regions that have been identified as being involved in intersubunit contact. Thus, it was proposed that these differences in sequence may determine the characteristic allosteric properties of each isozyme (Muirhead et al., 1986; Noguchi et al., 1986). Up to date, there are no data to elucidate the quantitative effects of the structural changes. Kinetic and structural studies on both the kidney and muscle PK isozymes were initiated. Results will be presented to identify the effects on the *global* behavior as a consequence of the localized structural difference between the isozymes.

Materials and Methods

Rabbit muscle (M_1) pyruvate kinase was obtained as an ammonium sulfate precipitate from Boehringer-Mannheim. The isozyme from rabbit kidney (M_2) was prepared essentially by the method of Ibsen et al. (1981). On staining of the SDS–PAGE with Coomassie Brilliant Blue, the M_1 and M_2 isozymes were >98% and >90% pure, respectively, as judged by densitometric scanning.

Tris-HCl, Tris base, the monopotassium salt of ADP, and the tricyclohexylammonium salts of PEP, FBP, NADH, and lactate dehydrogenase were obtained from Boehringer-Mannheim. KCl, $MgSO_4$, L-alanine, and succinimide were purchased from Sigma, and L-phenylalanine was from Schwarz/Mann.

Steady-state kinetic velocity was determined by the lactate dehydrogenase coupled enzyme assay (Buchler & Pfleiderer, 1955) as described by Oberfelder et al. (1984a). Under each experimental condition, e.g., at a specific inhibitor concentration, the initial velocity was measured at 16–20 different PEP concentrations ranging from 0 to 2 mM and 0 to 10 mM for M_1- and M_2-PK, respectively. Data were obtained as the change in absorbance at 340 nm/min. The maximal velocity (V_{max}) of each data set, which is expressed as substrate dependency of initial velocity, was determined by nonlinear curve fitting to the Hill equation as expressed in eq 1 (Hill, 1910).

$$v = \frac{V_{max}K^n[S]^n}{1 + K^n[S]^n} \quad (1)$$

The dependent variable, v, is the observed steady-state kinetic velocity, and V_{max} is the maximal velocity. The independent variable, [S], is the concentration of variable substrate, PEP. K is a complex steady-state kinetic equilibrium constant, and n is the Hill coefficient. This value of V_{max} was used to normalize each data set, enabling all of the data to be scaled between 0 and 1.0 (relative activity). This is accomplished to permit simultaneous analysis of all data sets. The fitting routine used for the data analysis was that of nonlinear parameter estimation based upon the algorithm of Marquardt (1963) and was described previously (Oberfelder et al., 1984b).

Since in previous studies all experimental data can be accommodated by a concerted, allosteric model (Oberfelder et al., 1984b), the kinetic data in this study are analyzed in a similar fashion.

The equation used in the simultaneous analysis is a modification of the two-state model of Monod et al. (1965) to account for the effects of both an allosteric inhibitor (Oberfelder et al., 1984b) and an activator, as shown in eq 2. This

$$v/V_{max} = \{k_2^R([S]/K_S^R)(1 + [S]/K_S^R)^3(1 + [I]/K_I^R)^4(1 + [A]/K_A^R)^4 + k_2^T L([S]/K_S^T)(1 + [S]/K_S^T)^3(1 + [I]/K_I^T)^4(1 + [A]/K_A^T)^4\}/\{(1 + [S]/K_S^R)^4(1 + [I]/K_I^R)^4(1 + [A]/K_A^R)^4 + L(1 + [S]/K_S^T)^4(1 + [I]/K_I^T)^4(1 + [A]/K_A^T)^4\} \quad (2)$$

equation contains three independent variables: [S], [I], and [A], which are the concentrations of the variable substrate PEP, the allosteric inhibitor Phe, and the allosteric activator FBP, respectively. The fitted parameters are as follows: k_2^R and k_2^T are the state-specific relative catalytic rate constants associated with the R and T states, respectively. k_2^T is determined from the relationship $k_2^T = (1.0 - k_2^R)$, due to the fact that the data were normalized to a maximum activity of 1.0. Hence, only k_2^R is allowed to float during data analysis. K_S^R and K_S^T, K_I^R and K_I^T, and K_A^R and K_A^T are the state-specific dissociation constants for substrate, inhibitor, and activator, respectively to the R and to the T state. Finally, L is the equilibrium constant that governs the state change and is defined as [T state]/[R state]. Hence, one set of eight independent parameters serves to define the steady-state kinetic velocity (in the presence of various substrate, inhibitor, and activator concentrations) for each isozyme.

Fluorescence lifetime measurements (Gratton et al., 1984; Lakowicz, 1983) were performed by multiple-frequency phase fluorometry at the Laboratory for Fluorescence Dynamics, Department of Physics, University of Illinois, Champaign–Urbana, IL. Phase angles and modulation ratios were measured with the fluorescence emission resulting from sample excitation at 295 nm (an interference filter, Hoya uv32, was used to block out scattered and emitted light below 320 nm; the emission maximum for the enzyme is 340–350 nm). Data were obtained for each sample at 13 logarithmically spaced frequencies, ranging from 2 to 250 MHz. All experiments were performed in TKM buffer at pH 7.5 and 23 °C. Measurements at each particular frequency were repeated until the standard error of the measurement was less than 0.2° for the phase angle shift and less than 0.004 for the modulation ratio. The reference compound was *p*-terphenyl in cyclohexane and was assumed to have a fluorescent lifetime of 1.0 ns. The frequency-dependent phase and modulation values were fitted to a multiexponential decay law (eq 3), where α_i and τ_i are

$$I(t) = \sum \alpha_i e^{-t/\tau_i} \quad (3)$$

the amplitude and decay time, respectively, of the ith com-

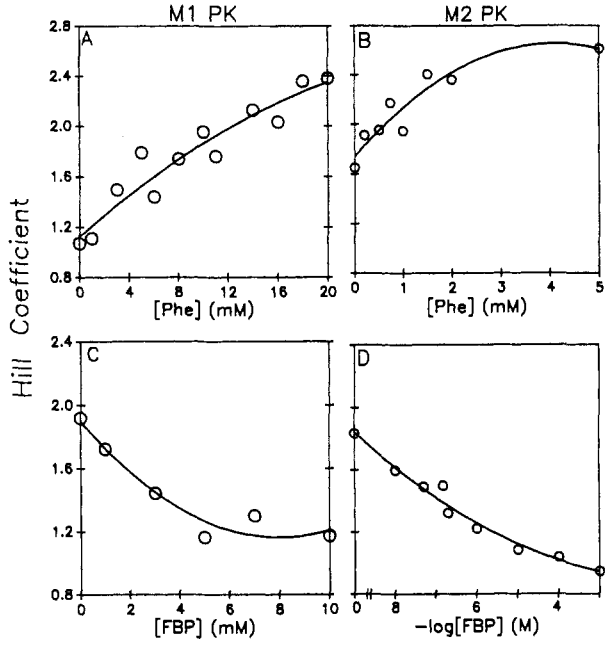


FIGURE 1: Effects of allosteric effectors on the Hill coefficient. Data for M_1-PK: (A) increasing concentration of Phe; (C) increasing concentration of FBP in the presence of 12 mM Phe. Data for M_2-PK: (B) increasing concentration of Phe; (D) increasing concentration of FBP in the presence of 1 mM Phe.

ponent. The fractional intensity (F_i) of each component of the emission is given by

$$F_i = \alpha_i\tau_i/\sum\alpha_i\tau_i \quad (4)$$

The effects of Phe on exposure of tryptophan residues in both isozymic forms of PK were monitored by measuring the quenching of the intrinsic fluorescence of these tryptophan residues by succinimide (Eftink & Ghiron, 1981). The excitation wavelength was 295 nm, and the emission was monitored at 335 nm on a Perkin-Elmer 512 double-beam spectrofluorometer. The sample solutions included PK at a final concentration of 75 μg/mL, along with appropriate amounts of succinimide and ligand, all of which were in TKM buffer at pH 7.5.

A constant for quenching can be obtained from the Stern–Volmer equation (Stern & Volmer, 1919):

$$F_0/F = 1 + K_q[Q] \quad (5)$$

where F_0 and F are the fluorescence intensities of the fluorophore in the absence and presence of succinimide, respectively, [Q] is the concentration of succinimide, and K_q is the weighted average of the collisional quenching constant, since PK contains three residues of tryptophan per subunit.

Results

In the PK system, it is conceivable that different isozymes are regulated by different mechanisms since the literature considers M_2-PK as allosteric whereas M_1-PK is not. Furthermore, M_1-PK has been reported not to be activated by FBP in the absence of inhibitor, yet M_2-PK is (Hall & Cottam, 1978; Ibsen & Trippet, 1973). To establish that the rabbit isozymes employed in this study do exhibit the same regulatory behaviors as that reported in the literature, steady-state kinetic data were collected for both M_1- and M_2-PK isozymes in the presence and absence of Phe or FBP. Using Phe as an inhibitor, it can be shown that inhibition by Phe resulted in sigmoidal substrate dependencies for both isozymes. This serves to illustrate the cooperativity inherent in the regulation of the enzymic activity. The cooperativity has been analyzed

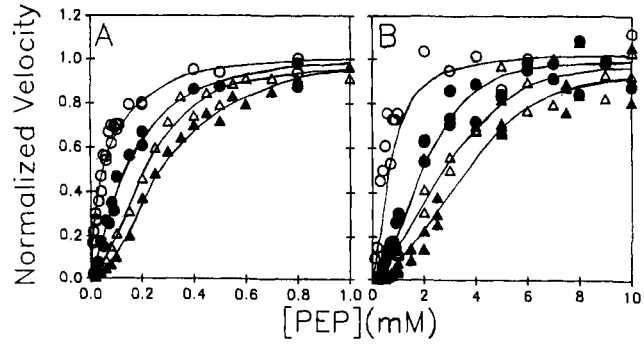


FIGURE 2: Relation between substrate concentration and enzyme activity of pyruvate kinase isozymes. Representative data sets in the presence of Phe are presented. (A) Data for M_1-PK. The symbols for Phe concentrations (mM) are (○) 0, (●) 3, (△) 10, and (▲) 16. (B) Data for M_2-PK. The symbols for Phe concentrations (mM) are (○) 0, (●) 0.5, (△) 1.0, and (▲) 2.0. The ADP concentration was 2.0 mM in all assays. The lines are drawn to indicate the trend only.

in terms of the Hill coefficient. Phe acts to increase the Hill coefficient for both isozymes, as shown in Figure 1A,B. In the case of the muscle isozyme, the value for this measure of cooperativity increases from 1.0 ± 0.1 to 2.4 ± 0.1 as the concentration of Phe is increased from 0 to 20 mM. The kidney isozyme is characterized by a Hill coefficient of 1.6 ± 0.1 in the absence of Phe, and the value of this parameter is increased to 2.7 ± 0.1 as the concentration of this allosteric inhibitor is increased to 5 mM. These results indicate that there is an inherent difference in the kinetic behavior of these two pyruvate kinase isozymes. M_2-PK exhibits a significant level of cooperativity, even in the absence of Phe, whereas M_1-PK exhibits this property only in the presence of the inhibitor. This result implies that in the absence of allosteric effectors these two isozymes may normally exist in different distributions between conformational states. Additional study also showed that rabbit M_2-PK is more susceptible to Ala inhibition than M_1-PK. Effects of activators on these isozymes were also studied, and the results demonstrate that both isozymes can be activated by FBP, although for M_1-PK the activating effect (or reversal of inhibition) can only be observed in the presence of inhibitor and the concentration of FBP required to activate this isozyme is significantly higher than that of M_2-PK. In the case of M_1-PK, as shown in Figure 1C as the concentration of FBP is increased, in the presence of a constant concentration of 12 mM the value for the Hill coefficient is decreased from 1.8 ± 0.1 to 1.0 ± 0.1. Similarly, the Hill coefficient for M_2-PK decreases from 1.7 ± 0.1 to 1.0 ± 0.1 as the concentration of FBP is increased, as shown in Figure 1D. These results infer the existence of an interconversion between alternate conformers for both pyruvate kinase isozymes being studied.

Having established that both isozymes can be activated or inhibited by FBP and Phe, respectively, the basic kinetic behavior of these isozymes should be quantitatively evaluated in order to identify the particular equilibrium constant(s) that is (are) altered as a consequence of structural change. To accomplish such a goal, it is imperative that information spanning different regimes of ligand concentration ranges should be obtained. Hence, extensive steady-state kinetic measurements were conducted. For M_1-PK, kinetic data were collected at 25 conditions constituted by various combinations of five concentrations of Phe and FBP; e.g., at a fixed Phe concentration of 12 mM, five data sets were collected at five FBP concentrations ranging from 0 to 10 mM. For each set of fixed concentrations of Phe and FBP a curve of activity vs PEP concentrations was obtained with 16–20 different PEP

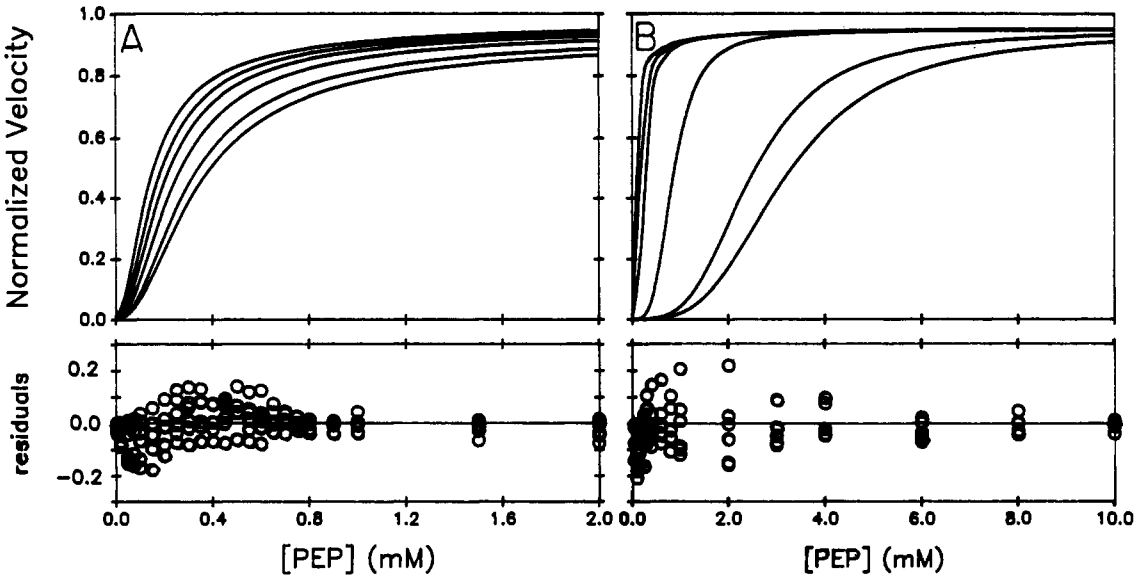


FIGURE 3: Relation between substrate concentration and enzyme activity. Representative fitted curves illustrate the activation and inhibition afforded by the allosteric effectors of the muscle (A) and the kidney (B) isozymes of pyruvate kinase. The muscle isozyme data sets were measured in the presence of 12 mM Phe and as a function of FBP: 0, 1, 3, 5, 7, and 10 mM. The kidney isozyme data were measured in the presence of 2 mM Phe and as a function of FBP: 0, 0.01, 0.1, 0.5, 1, and 10 μM. The lines were drawn on the basis of the nonlinear parameter estimates. Data points were omitted for clarity. The residuals of the fit are displayed below the substrate dependency.

Table I: Summary of the Equilibrium and Kinetic Parameters for the Pyruvate Kinase Isozymes

	M_1-PK	simulated error	M_2-PK
L	0.06 ± 0.04	0.50–1.0	800 ± 530
K_S^R	60 ± 6.8	0.03–0.07	64 ± 9.3
K_S^T	480 ± 29	0.05–0.07	7000 ± 3100
K_I^R	13 000 ± 5800	0.08–0.16	850 ± 330
K_I^T	780 ± 320	0.20–0.34	90 ± 24
K_A^R	10 000 ± 3400	0.02–0.17	0.06 ± 0.02
K_A^T	>50 000	0.10–0.50	1.3 ± 0.27
k_2^R	1.0 ± 0.02	0.01–0.02	0.97 ± 0.01
k_2^T	0.00		0.03

[a] L is a unitless parameter. The K values are expressed in micromolar, and the k values are expressed as relative activity, 1.0 being equal to V_{max}. The errors associated with the parameter estimates were derived from standard deviations of multiple fittings of various subsets of the data and with different initial estimates for some parameters. The errors for each individual fitting were usually smaller than the value shown and were similar in magnitude to the error estimates from simulations on the recovery of parameters values, shown in the center column (see text).

concentrations; i.e., over 500 data points were employed for simultaneous data analysis. Panels A and B of Figure 2 show a representation of the results. The effects of the allosteric ligands were analyzed quantitatively in terms of dissociation constants for enzyme–ligand interactions, by subjecting this multidimensional steady-state kinetic data base to simultaneous nonlinear parameter estimation. The data were fit to eq 2, which describes a two-state model of allosteric regulation involving both an allosteric activator and an inhibitor (Monod et al., 1965; Oberfelder et al., 1984b). Typical results of this analysis are shown in Figure 3. It is evident that the residuals are randomly distributed. The various equilibrium constants obtained from this analysis are summarized in Table I. The error associated with the values of the estimated parameter is derived from standard deviations. It is important to note that the various equilibrium constants derived for the muscle enzyme in this study are in very good agreement with the results of an independent study 5 years ago (Oberfelder et al., 1984b).

On the basis of the results of data fitting, one of the states is active and the other is inactive. This has been supported by parameter estimation of the relative catalytic rate constants for the enzyme reaction, k_2. The k_2 value for one of the states, arbitrarily designated (traditionally) as the R state, is responsible for >95% of the catalytic activity, whereas the other conformation, the T state, is relatively inactive.

One of the more significant differences between the two isozymes studied, in terms of the fitted parameters, was in the value estimated for L, the intrinsic equilibrium constant that governs the state change between the active and inactive conformations of the enzyme in the absence of ligands. The value for this constant differs by several orders of magnitude between M_1-PK and M_2-PK. This equilibrium for the muscle enzyme favors the active conformation, whereas in the case of the kidney enzyme the inactive conformation is the predominating species. If the difference in L alone can account for the observed difference in the kinetic behavior for the two isozymes (i.e., M_1-PK and M_2-PK exhibit hyperbolic and sigmoidal substrate dependency, respectively), then one can expect to fit the results for M_2-PK by using the dissociation constants determined for M_1-PK with the exception of L. The expected fit should show no systematic deviation. The results of such a test show systematic deviation, thus, the difference in L alone cannot account for the difference in kinetic behavior between the two isozymes.

The dissociation constants estimated for the binding of PEP to the R state of PK, K_S^R, are seen to be similar for both isozymes; both exhibit an R-state affinity for this substrate of about 60 μM. PEP binds preferentially to the R state by at least 1 order of magnitude in each case. The binding of substrate to the T state, K_S^T, is reduced significantly for the kidney isozyme as compared to that for the muscle isozyme, e.g., 7000 μM as compared to 480 μM.

The differential sensitivity of the two isozymes to Phe inhibition noted earlier is due, in part, to the magnitude of the state-specific dissociation constants for the binding of Phe, K_I^R and K_I^T. Both isozymes exhibit preferential binding of Phe to the T state by about 1 order of magnitude. The consequence is that Phe binds to the T state and shifts the distribution of the PK conformation toward the T state. This, coupled to the fact that the T state is relatively inactive, leads to the inhibitory effect observed for this allosteric ligand. In addition, regardless

of the conformation state, the kidney enzyme binds Phe with at least 8-fold higher affinity than the muscle enzyme. Thus, the higher affinity for Phe by the T state which is inactive renders M_2-PK more susceptable to Phe inhibition.

The state-specific dissociation constants, K_A^R and K_A^T, governing the binding of FBP were also estimated from the steady-state kinetic data. It is readily apparent that there is a large difference in the affinity of the isozymes for this allosteric activator. The kidney isozyme exhibits high affinity, whereas the muscle enzyme has a much weaker binding constant (millimolar range). In both cases, however, the affinity for the R state is greater than that for the T state. For the muscle isozyme, this parameter (K_A^T) is unmeasurable; it approaches infinity as the fit converges. The differential sensitivity afforded by the difference in state-specific binding constants for this activator is responsible for the allosteric activation that is observed experimentally. M_2-PK has higher affinity for FBP, and the active R state preferentially interacts with FBP; thus, in the presence of FBP, the distribution of the M_2-PK conformation is in favor of the active R state. These results provide a logical rationale for the observed higher sensitivity of M_2-PK to FBP activation.

Although nine different constants are employed to fit the kinetic results, the assignment of values to these constants is not arbitrary. The validity of the analytical procedure employed in this study has been tested quite successfully in an earlier study using not only kinetic but also ligand binding and structural data (Oberfelder et al., 1984b). Since the specific values of equilibrium constants are the basis for further development of regulatory mechanism in this study and steady-state kinetic data will be the *only* data base employed, it is imperative to establish the accuracy of this quantitative analysis with only data from steady-state kinetics. The kinetic data required in this study on M_1-PK were analyzed, and the equilibrium constants were compared with those that were determined with the benefit of ligand binding and structural data. It is most worthy to note that the various constants derived for M_1-PK in this study are in good agreement with the results of an earlier study (Oberfelder et al., 1984b). Thus, it can be concluded that kinetic data alone, at least in the present case, can yield the same equilibrium constants as compared to those that are derived from a greater variety of data including ligand binding and structural information. As an additional test on the validity of the quantitative assignment of these various constants, simulations were performed to test the confidence level of parameters by the fitting procedures in a manner analogous to that of Johnson and Frasier (1985). It was found that all of the parameters were recovered with reasonable certainty but that some were more reliable than others. The parameters that were most accurately recovered were those that are most directly assayed by the experimental measurement, those being the relative catalytic rate constants k_2^R and k_2^T and the binding constants for the substrate, PEP (K_S^R and K_S^T). The standard deviation of the independent simulations for these parameters was less than 7%, and the accuracy was more than 97% (i.e., ±3% of the starting parameter values). The other state-specific binding constants, those for the binding of Phe (K_I^R and K_I^T) and FBP (K_A^R and K_A^T), were recovered with a slightly greater standard deviation of 10–20% and an accuracy of ±5–10%. The least well recovered parameter was the equilibrium constant governing the state change, L, which often varied by 50–100% of the starting value. It seems that the recovery of this parameter is limited to an approximation of within an order of magnitude. However, even at this level of estimation, the results provide some useful information concerning the global behavior of the enzymes as a consequence of changes in primary sequences, especially in light of the direct conformational studies reported herein and elsewhere (Oberfelder et al., 1984a; Consler & Lee, 1988; Consler et al., 1988).

The basis for the two-state model being tested is that the enzyme can exist in alternative states and that these two states have differential affinities toward the substrates and/or effectors. Evidence for their existence and the structural characterization of these alternate states have been reported for the M_1 isozyme (Oberfelder et al., 1984a,b; Consler & Lee, 1988; Consler et al., 1988). Analogous structural data for the M_2 isozyme is not available; therefore, experiments were conducted in an effort to provide structural information for the M_2 isozyme. Due to the limited quantity of the M_2 isozyme available, a technique was chosen that would allow a reasonable signal at low concentrations of protein. The intrinsic fluorescence of tryptophan residues is ideally suited for such a study. In particular, the determination of the excited-state fluorescent lifetimes of tryptophans in these PK isozymes may yield evidence for the existence of alternate states in both of these enzymes.

In order to test this technique for its usefulness in describing the conformation of the PK isozymes, the measurements were first carried out on the M_1-PK isozyme, for which the conformation has been well studied. The fluorescence excited-state lifetime data measured (phase angle shift and modulation ratio) *cannot* be fit by single-component analysis since the χ^2 values were >200. It was best fit by three-component analysis, whether the model used to describe the fluorescence decay was simple exponential or one of several population distributions. The choice of three-component over two-component analysis is dictated by the significant improvement of the x^2 value changing from >5 to <3. In all cases the analysis yielded one minor lifetime component (fraction less than 10%) with a magnitude of less than 1 ns. Each of the other two lifetimes was found to comprise 40–60% of the fluorescence emission signal. The second lifetime was determined to be 2–3 ns, and the third lifetime value varied between 5 and 8 ns, depending upon the sample conditions. It is this third lifetime which apparently provides information for the conformational states of PK. In the presence of buffer alone or in the presence of substrate, PEP, or activator, FBP, this parameter had a value of 5 ns. M_1-PK is known to assume the "active" R state under all of these conditions. It is interesting to note that both PEP and FBP do not cause any shift in the lifetime measurements; thus, these results imply that these ligands do not induce a shift in the conformation of M_1-PK. This observation is in total agreement with the kinetic measurement of L; i.e., M_1-PK is basically in the R state, and PEP or FBP will not perturb the distribution between R- and T-state under these conditions. In the presence of Phe, however, this lifetime was shifted to a longer lived component of 7 ns; thus, this result implies that the environments surrounding the tryptophan residues are perturbed by the binding of Phe. These results provide another insight into the conformational changes involved in the conversion of the R to the T state in M_1-PK. Knowing that Phe acts to shift the equilibrium between the alternate states of M_1-PK toward the "inactive" T state, one may conclude that the T state assumes a more asymmetric structure as revealed by hydrodynamic techniques (Oberfelder et al., 1984a; Consler et al., 1988) and that the tryptophan residues of that structure exhibit a longer lived lifetime. The fact that PEP and FBP do not have any effect on the lifetime measurements while Phe does indicates that fluorescence lifetime measurements can

Table II: Summary of the Fluorescence Lifetimes of Tryptophan Emission in the Pyruvate Kinase Isozymes: Discrete Exponential Analysis

exptl condition	no. of exponentials	T_1	F_1	T_2	F_2	T_3	F_3	χ^2
				M_1-PK				
5 mM FBP	1	3.5						225.8
	2	4.4	0.90	0.9	0.10			4.6
	3	5.3	0.61	2.6	0.36	0.2	0.03	0.6
TKM	1	3.2						337.4
	2	4.5	0.84	1.0	0.16			5.0
	3	5.2	0.57	2.4	0.37	0.5	0.06	3.1
12 mM Phe	1	4.6						279.8
	2	4.9	0.75	0.9	0.25			64.1
	3	6.9	0.52	2.5	0.44	0.4	0.05	0.8
				M_2-PK				
0.1 mM FBP	1	3.3						482.9
	2	4.7	0.85	0.9	0.15			8.6
	3	5.9	0.50	3.2	0.41	0.59	0.08	3.7
TKM	1	2.7						556.8
	2	4.7	0.74	1.1	0.26			16.1
	3	6.6	0.45	2.1	0.51	0.001	0.04	0.8
3 mM Phe	1	3.0						559.8
	2	5.0	0.76	1.1	0.24			13.5
	3	8.5	0.29	3.3	0.60	0.7	0.11	2.5

[a] Fluorescence lifetimes, T_i, are expressed in nanoseconds, and the fractional contribution, F_i, of each component is given. Reduced χ^2 values for each fit are shown to the right.

provide information on the shift in the equilibrium distribution of R and T states of M_1-PK. These data are illustrated in Figure 4 and summarized in Table II.

Having obtained a qualitative correlation between fluorescence and other methods on the studies of M_1-PK, the same analysis is applied to the M_2-PK. Consistent with the data analysis for the M_1-PK isozyme, the analysis of the data for the M_2-PK isozyme showed that the longest lived fluorescent excited-state component may be used as an indicator of the conformation of the enzyme. It was seen that M_2-PK in the presence of buffer alone was characterized by a 7-ns fluorescent lifetime. In the presence of FBP this component was shifted to a shorter value, indicating that this activator does have an influence on the conformation of the enzyme in contrast with the results for M_1-PK. It is important to note that for M_2-PK FBP can induce a change in the conformation of the protein whereas FBP has no observable effect on M_1-PK. This differential ability for FBP to shift the fluorescent signal is in good agreement with the observed values of L. For M_2-PK, the distribution of states is in favor of T states. FBP, having greater affinity for the R state, would bind favorably to the R state of M_2-PK and shift the distribution toward the R state, as reflected by the change in fluorescence signal. For M_1-PK, the distribution of states is highly in favor of the R state; hence, FBP cannot exert much effect on the state change and resulted in no observable change in fluorescence signal. On the other hand, Phe exhibits a similar effect on both isozymes, and the specific nature of the signal change is to increase the lifetime instead of to decrease it as observed in M_2-PK in the presence of FBP. Since in both isozymes the distribution of states is such that Phe can exert a shift toward the T state, it is logical that Phe can affect the fluorescence signal for both isozymes.

These results are important for several reasons; they indicate (1) that the fluorescence excited-state lifetimes may be used to probe the global conformation of PK isozymes in the absence of any other structural information, (2) that both isozymes do exist in different conformational states, the distribution of which is influenced by activator and inhibitor, and (3) that the equilibrium governing the state change for the M_1 and M_2 isozymes in the absence of ligands favors the R and T state, respectively. These conformational data are

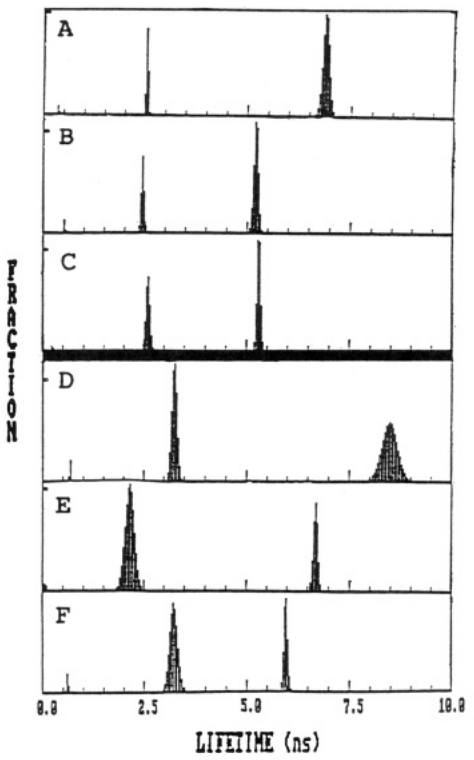


FIGURE 4: Fluorescence lifetime distributions for the pyruvate kinase isozymes. Three-component analysis using a Gaussian population distribution model function is shown for the muscle (A–C) and the kidney (D–F) pyruvate kinase isozymes. Data were obtained in TKM buffer alone (B and E), in the presence of the allosteric activator, FBP (C and F), and in the presence of the allosteric inhibitor, Phe (A and D). Similar results were obtained for other three-component analyses, including an exponential and a Lorentzian population model; these are summarized in Table II.

entirely consistent with our previous studies on the M_1-PK isozyme (Oberfelder et al., 1984a,b; Consler et al., 1988). They also support the parameter estimates for L, which were reported above for both PK isozymes (analysis of the steady-state kinetic data).

The conformational transition is the underlying basis for the model of allosterism being tested; therefore, it was of interest to further characterize the structure of these two

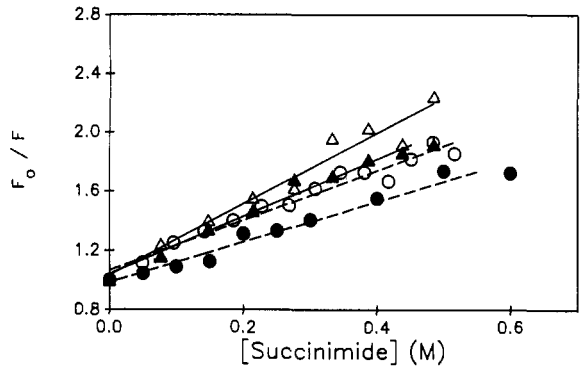


FIGURE 5: Stern–Volmer plots of succinimide quenching data. Circles represent data for M_1-PK, and triangles are those for M_2-PK. Open and filled symbols represent data obtained in the absence and presence of Phe, respectively. The concentration of Phe used was 12 and 3 mM for the M_1- and M_2-PK isozymes, respectively. Lines were drawn on the basis of linear regression of each data set.

isozymes. Initially, partial proteolytic digestion was used in an effort to probe for structural information, as this method has been used previously for the characterization of the structural transition in the muscle isozyme (Consler & Lee, 1988). However, these digests indicated that there must be a structural difference in the kidney isozyme. No conditions were found that resulted in the digestion of this isozyme into discrete peptides, despite altering the protease, ligands, temperature, and ionic strength of the experiment. The kidney isozyme seems to be extremely susceptible to proteolytic digestion; once cleaved by protease, the remaining polypeptides are rendered protease sensitive, resulting in complete digestion of the polypeptide. Additional information on the conformational properties of these isozymes was sought by quenching of intrinsic protein fluorescence with succinimide, a quencher that is larger in size than acrylamide, which was found to be too small to be discriminated by PK in the presence of various ligands (Oberfelder, 1982). Each of these isozymes contains three tryptophans per subunit, and these are among the identically conserved residues. The results of these experiments are summarized in Figure 5. In the presence of buffer alone, the weight-average quenching constant, K_q, was 1.7 ± 0.2 and 2.5 ± 0.2 M^{-1} for M_1- and M_2-PK, respectively. The higher the value for this "collision-association" constant, the more accessible are the tryptophan residues to the quenching agent. These results imply that the kidney isozyme is more susceptible to the action of a collisional quencher potentially as a consequence of a significant increase in dynamic motions or simply greater exposure of the tryptophan residues. Combining this result with that from the proteolytic digestion implies that an increase in dynamic motions could be the more appropriate interpretation.

In addition, the effect of Phe on succinimide quenching was determined. For the muscle isozyme in the presence of 12 mM Phe, the value for K_q was reduced to 1.1 ± 0.2 M^{-1}, and for the kidney isozyme in the presence of 3 mM Phe, the corresponding value was decreased to 2.0 ± 0.2 M^{-1}. In both systems the effect of Phe is to reduce the extent of collisional quenching, implying that this ligand has some impact upon the conformation of the enzyme, effectively changing at least the local environment of tryptophan residues in the protein from a more exposed state to a lesser one. A preliminary hydrogen exchange experiment showed that the rate of exchange is slower in the presence of Phe. Hence, at present all experimental data pertaining to protein dynamics imply that the kidney isozyme exhibits different dynamic behavior and the presence of Phe perturbs it.

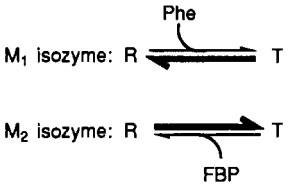
Discussion

The results presented herein describe the structure and activity of two of the four known mammalian PK isozymes, those from skeletal muscle (M_1-PK) and kidney tissue (M_2-PK). These two isozymic forms were chosen for this study because it had been reported that in rat these two isozymes arise as a result of alternative splicing of a precursor mRNA transcribed off of a single gene (Noguchi et al., 1986). More importantly, the alternative splicing involved the exchange of only a single exon, each encoding 45 amino acids. Furthermore, the exchanged exons differed in the encoding of only 21 of these residues, and half of those were seemingly conservative substitutions. The remainder of the 530 amino acid residues encoded by the two mRNAs are identical. Thus, there is greater than 95% sequence identity between these two isozymes, including all of the residues that have been identified as being at or near the active site region of the cat muscle enzyme, for which the structure has been solved (Stuart et al., 1979). The muscle isozymes from various mammalian species (i.e., cat, rat, and rabbit) exhibit a similar extent (>95%) of sequence identity between themselves.

The primary sequence coded by the single exon is located at the Cα1 and Cα2 helical regions, one of the structural regions that Muirhead and co-workers have localized as responsible for intersubunit contact (Stuart et al., 1979; Muirhead et al., 1986). This region is separated from the active site by the A domain; thus, a very interesting issue is whether the Cα1 and Cα2 regions exert any influence on the rest of the enzyme molecule via long-range effects. Another most interesting issue is to define the specific influence of this structural change on the various equilibrium reactions that constitute the regulatory mechanism of PK. Since the structural perturbation is localized at the intersubunit contact, intuitively it is logical to ascribe the effect of this structural change to be correlated to subunit communication and in turn to a change in L only. However, results from this study demonstrate that, in contrast to the expectation, a change in primary sequence in the contact region leads to changes in six of the seven equilibrium constants. These results, thus, imply that a change in the primary structure in the Cα1 and Cα2 regions influences *not only the local environment but that its effects are propagated through long distance*, influencing remote parts of the protein molecule. This conclusion is consistent with the drastic changes in proteolytic susceptibility and accessibility of tryptophan residues observed in M_2-PK. Of course, the accuracy and validity of these equilibrium constants need to be verified by other approaches, one of which is direct measurement of global structural changes as a function of ligand concentrations. Recent preliminary results determined by analytical column chromatography are in good agreement with the predicted results based on the equilibrium constants shown in Table I (Heyduk and Lee, unpublished results).

The experimental results reported in this study serve to define the effects that the amino acid substitutions have upon the structure and function of the two PK isozymes under investigation. Both isozymes are subjected to qualitatively similar modes of allosteric regulation, and these interactions can be described with a simple two-state model of allosterism; the enzyme exists in R and T states, the distribution of which is governed by an equilibrium. These two states exhibit different affinities for substrate and effectors. The primary differences between the isozymes are in the quantitative assignment of values for the equilibrium constants governing the various interactions underlying the steady-state kinetic be-

havior. At present, there is no need to invoke additional complexity to the model by including additional interactions or changing the nature of the interactions, e.g., additional conformational states. These isozymes are different only due to small perturbations at key structural locations, which in turn lead to changes in *not* one specific equilibrium constant but in a majority of the equilibrium constants governing the basic behavior of the enzymes. As a consequence of these changes, the isozymes exhibit quantitatively different kinetic behaviors under the same experimental conditions. A simplified representation of the proposal is shown in eq 6, where R and T



(6)

represents the active and inactive forms, respectively, and the thickness of the arrow denotes the species favored by the chemical equilibrium. The structural elements which are responsible for the change in chemical equilibrium and which are most likely involved in subunit contacts were identified as the N-terminal domain (residues 10–40) and helixes 1 and 2 of domain C (residues 385–425). Evidence in support of this assignment is ample in the literature, and it can be shown that these elements of structure do differ among the isozymic forms of PK. It can be imagined that these structural units play an important role in intersubunit communication and thus are responsible for the transmission of information between the subunits. They constitute the switching mechanism between alternate conformations of PK. If, indeed, this structural region plays an important role in conferring specific allosteric behavior in PK, then one might expect that this region will be sensitive to mutation. Recently, it was reported that the two other forms of rat pyruvate kinase [the liver (L-PK) and erythrocyte (R-PK) isozymes] arise as a result of differential transcripts which are encoded by the same gene (Noguchi et al., 1987). Thus two genes encode all four pyruvate kinase isozymes, in a pairwise fashion. As with M_1- and M_2-PK, the difference between L-PK and R-PK is a limited exchange of a small number of amino acid residues in a localized region. This localized region again corresponds to one of the consensus regions of intersubunit contact that were identified for the cat muscle enzyme structure. In this case the region of structure that is affected is the N-terminal domain. This structural element is in close interaction with the Cα1 Cα2 helixes and helps to form the network of subunit interactions between the four identical subunits of the tetramer. In addition, the catalytic activity of the L isozyme is known to be allosterically regulated by phosphorylation [reviewed by Engstrom (1978)]. The site of this covalent modification is Ser12 (Hjelmquist et al., 1974), which is precisely in the N-terminal domain of the enzyme. The fact that M_1- and M_2-PK lack that sequence extention in the N-terminus explains why these two isozymes are not phosphorylated and thus are not regulated by this chemical modification (Noguchi et al., 1987).

It is becoming clear that differences in a localized region in the sequence and structure of PK, the intersubunit contact region, confer characteristic properties on each of the four PK isozymes. The perturbation of this subunit interface by amino acid substitution, by covalent posttranslational modification, or by alteration of solution conditions leads to an allosteric transition between conformers of the enzyme. These states each have characteristic catalytic properties and thus allow for precise regulation of pyruvate kinase activity in a tissue-specific manner. A unifying working hypothesis is therefore proposed for the regulation of pyruvate kinase, that being based upon the existence of a characteristic allosteric transition between alternate states of the isozyme. These changes in primary sequence also effect the various equilibrium reactions in spite of the fact that there is a great majority of sequence identity among these isozymes.

In recent years, the interest in the effects of amino acid substitution on protein structure and function has grown tremendously (Ackers & Smith, 1985; Benkovic et al., 1988; Knowles, 1987). With the advent of site-specific mutagenesis techniques which allow for the replacement of amino acid residues within a protein, virtually at will, the importance of these long-range effects has become even more apparent. Clearly, it is necessary to improve the understanding of how amino acid changes can affect the overall structure and function of a protein. By studying these effects in naturally altered proteins (mutants and isozymes) in combination with those obtained artificially in the laboratory, one can now hope to achieve this goal sometime in the not too distant future.

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Substrate Recognition Determinants for Rhodopsin Kinase: Studies with Synthetic Peptides, Polyanions, and Polycations†

Krzysztof Palczewski,‡ Anatol Arendt,‡ J. Hugh McDowell,‡ and Paul A. Hargrave*,‡,§
Department of Ophthalmology and Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, Florida 32610

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ABSTRACT: Rhodopsin kinase phosphorylates serine- and threonine-containing peptides from bovine rhodopsin's carboxyl-terminal sequence. K_m's for the peptides decrease as the length of the peptide is increased over the range 12-31 amino acids, reaching 1.7 mM for peptide 318-348 from the rhodopsin sequence. The K_m for phosphorylation of rhodopsin is about 10^3 lower than that for the peptides, which suggests that binding of rhodopsin kinase to its substrate, photolyzed rhodopsin, involves more than just binding to the carboxyl-terminal peptide region that is to be phosphorylated. A synthetic peptide from the rhodopsin sequence that contains both serines and threonines is improved as a substrate by substitution of serines for the threonines, suggesting that serine residues are preferred as substrates. Analogous 25 amino acid peptides from the human red or green cone visual pigment, a β-adrenergic receptor, or M_1 muscarinic acetylcholine receptors are better substrates for bovine rhodopsin kinase than is the peptide from bovine rhodopsin. An acidic serine-containing peptide from a non-receptor protein, α_{s1}B-casein, is also a good substrate for rhodopsin kinase. However, many basic peptides that are substrates for other protein kinases—histone IIA, histone IIS, clupeine, salmine, and a neurofilament peptide—are not phosphorylated by rhodopsin kinase. Polycations such as spermine or spermidine are nonessential activators of phosphorylation of rhodopsin or its synthetic peptide 324-348. Polyanions such as poly(aspartic acid), dextran sulfate, or poly(adenylic acid) inhibit the kinase. Poly(L-aspartic acid) is a competitive inhibitor with respect to rhodopsin (K_I = 300 μM) and shows mixed type inhibition with respect to ATP.

Rhodopsin is the photoreceptor protein of rod cells in the vertebrate retina. As part of the visual transduction process, rhodopsin becomes phosphorylated by a specific protein kinase, rhodopsin kinase. This phosphorylation following light absorption is one mechanism of terminating the excitation process [reviewed by Stryer (1986)]. Receptor phosphorylation may be a general process for receptor deactivation in other signal transduction systems. A number of other receptor proteins have been found to be homologous to rhodopsin and have been shown to undergo phosphorylation [reviewed by Sibley et al. (1987); Dohlman et al., 1987].

The phosphorylation of rhodopsin by rhodopsin kinase has been extensively studied [reviewed by Hargrave (1982) and Kühn (1984); Hargrave et al., 1988]. Prior to its exposure to light, rhodopsin is not a substrate for rhodopsin kinase. Following light exposure, rhodopsin undergoes a change in conformation to form metarhodopsin II, which is a substrate for the kinase. Phosphorylation occurs on serines and threonines located mostly in a compact region of rhodopsin's carboxyl-terminal sequence (Hargrave et al., 1980; Thompson & Findlay, 1984).

The sites of phosphorylation for many protein kinase substrates have been identified and their sequences determined. Such sequence information and the use of synthetic peptides as substrates have led to an understanding of the recognition determinants for many protein kinases. Extensive studies have been performed for determination of substrate specificity for cAMP and cGMP protein kinases, protein kinase C, and casein kinase II, among others (Kemp et al., 1975, 1976, 1977; Glass & Krebs, 1979; Kuenzel & Krebs, 1985; Kuenzel et al., 1987; House et al., 1987; Hassell et al., 1988). In preliminary experiments we showed that rhodopsin kinase could phosphorylate synthetic peptides containing the phosphorylation sites

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‡ Department of Ophthalmology.
§ Department of Biochemistry and Molecular Biology.

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