The Negative Dominant Effects of T340M Mutation on Mammalian Pyruvate Kinase*

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A fundamental issue in allosteric regulatory enzymes is the identification of pathways of signal transmission. Rabbit muscle and kidney pyruvate kinase isozymes are ideal to address this issue because these isozymes exhibit different enzymatic regulatory patterns, and the sequence differences between these isozymes have identified the amino acid residues that alter their kinetic behavior. In an earlier study, Cheng et al. (Cheng, X., Friesen, R. H. E., and Lee, J. C. (1996) J. Biol. Chem. 271, 6313–6321), reported the effects of a threonine to methionine mutation at residue 340 in the muscle isozyme. In this study, the same mutation was effected in the kidney isozyme. Qualitatively, the same negative effects are observed in both isozymes, namely a significant decrease in catalytic efficiency and decrease in apparent affinity for phosphoenolpyruvate but no change in affinity for ADP, and a decrease in responsiveness to the presence of effectors, be it activator or inhibitor. Because the diversity in the primary sequence between these two isozymes does not alter the negative impact of the T340M mutation, it can be concluded that this mutation exerts a dominant, negative effect. The negative effects of T340M mutation on the kinetic properties imply that there is communication between residue 340 and the active site. Residue 340 is located at the 1,4 subunit interface; however, a T340M mutation enhances the dimerization affinity along the 1,2 subunit interface. Thus, this study has identified a communication network among the active site, residue 340, and the 1,2 subunit interface.

Mammalian pyruvate kinase (ATP:pyruvate 2-O-phosphotransferase, EC 2.7.1.40) (PK) is a key regulatory glycolytic enzyme which catalyzes the transphosphorylation from phosphoenolpyruvate (PEP) to ADP. One of the enzymatic products, pyruvate, is situated at a major metabolic junction between the phosphoenolpyruvate (PEP) to ADP. One of the enzymatic products, pyruvate, is situated at a major metabolic junction between the metabolic pathways of carbohydrates, amino acids, and lipids. Thus, a tight regulatory control of PK is critical to proper cellular function.

PK activity in mammalian cells is regulated by two different mechanisms. The first mechanism occurs at the level of expression. The mammalian genome contains two distinct genes coding for four different enzymes with PK activity. The M1- and M2-type isozymes of PK are produced from one gene by alternative RNA splicing (1). The R- and L-type PK isozymes are products of a different gene with two different promoters. The R- and L-type mRNAs differ only in their 5'–terminal sequences (2). These four isozymic forms of PK are expressed in a tissue-specific manner (3). The M1-type PK is the major isozyme of cardiac muscle and brain and is the only isozyme found in adult skeletal muscle. The M2-type PK is widely distributed throughout the body and is the major isozyme derived from kidney and leukocytes. The L-type PK is the major isozyme in the liver, and the R-type isozyme is isolated from erythrocytes.

The second mechanism of control of PK activity is through allosteric regulation. PK isozymes are regulated by a host of allosteric inhibitors and activators (3). The M1- and L-type isozymes are homotropically activated by PEP and heterotropically activated by fructose-1,6-bisphosphate (FBP). The M2-type isozyme is activated by PEP and FBP only in the presence of the inhibitor i-phenylalanine (Phe), which is an inhibitor of all isozymes. In addition, the catalytic activity of the L-type isozyme is known to be allosterically regulated by phosphorylation (4). The four PK isozymes have distinct kinetic characteristics. Thus, these isozymes are genetic products to accommodate the various metabolic requirements demanded by the different tissues.

In general, the current knowledge of the molecular mechanisms of allosteric regulation at the atomic level is lagging far behind that of the molecular mechanism of catalysis. Whereas a catalytic site usually comprises a part of the enzyme, allosteric control is transmitted over a long range, thus significantly increasing the number of possible residues involved in regulation. It is, therefore, difficult to identify the structural elements that play an important role in the regulation of PK and the propagation of these allosteric signals. Recently, an approach was successfully applied to increase the efficiency of identifying structural elements involved in the regulation of PK (5). The approach is based on the assumption that the molecular mechanism of allosteric regulation is conserved for all mammalian PK isozymes. This hypothesis is based on the structural homology among mammalian PK isozymes and their qualitatively similar functional behavior. It follows from this hypothesis that structural elements involved in the regulation of PK have similar functional significance in the other mammalian PK isozymes. Guided by this hypothesis, genetic information from different PK isozymes is collected to identify the targets for mutational studies. Thus far, 55 different mutations have been characterized in the human R-type PK isozyme from patients with PK-deficient hemolytic anemia (6). Mutant PK from a patient with PK-deficient hemolytic anemia was identified by genetic studies of...
K421 (1,2 subunit interface) is 39 Å, and between K421 and the active molecule in the active site is 24 Å, between the T340M mutation and the pyruvate different between RMPK and RKPK is in black gray respectively. The T340M mutation site in each subunit is marked. The two interface (Biochemistry 37, T340M mutation.2 The T340M rRKPK mutant is characterized in the presence of a background of sequence differences relative to the active site and the subunit interfaces. The characterization in a Sorvall RC-5C centrifuge using the GS-3 rotor at 6000 rpm for 20 min.

Fig. 1. Schematic diagram of the T340M rRKPK tetramer looking down two 2-fold axes, constituting the 1,4 intersubunit interface (A-AXIS) and the 1,2 subunit interface (B-AXIS), respectively. The T340M mutation site in each subunit is marked. The two pairs of dimer are in different shades of gray. The sequence that is different between RMPK and RKPK is in black along the B axis. The approximate distance between the T340M mutation and the pyruvate molecule in the active site is 24 Å, between the T340M mutation and K421 (1,2 subunit interface) is 39 Å, and between K421 and the active site is 45 Å. These distances are based on the x-ray crystallographic structure of RMPK (11).

eythrocyte PK in hereditary nonspherocytic hemolytic anemia patients (7–9). This mutant involves a threonine to methionine at a residue equivalent to 340 in rabbit kidney PK (RKPK). Threonine 340 or its equivalent is conserved in all known aligned PK sequences (10). Based on the crystal structure of RMPK, it is located outside of the active site (11). Fig. 1 shows a schematic representation of the location of T340M mutation relative to the active site and the subunit interfaces. The characterization of the T340M mutation in RMPK has been reported (5). Characterization of the same mutant in the rRKPK will not only provide information to address the validity of the hypothesis of an evolutionary conserved allosteric mechanism, it will also provide information on the dominance of this mutation in the presence of a background of sequence differences between rRKPK and rRMPK. The ability of rRKPK to undergo subunit assembly provides a unique opportunity to monitor the changes in energetics at the subunit interface as a result of the T340M mutation.2 The T340M rRKPK mutant is characterized by kinetic and physical studies. The results are analyzed to define the sites that communicate with each other, and a structural model for the transmission of the allosteric signal is discussed.

Experimental Procedures

Materials—Lactate dehydrogenase, disodium salt of ADP, phosphoenolpyruvate, Tris base, and Tris-HCl were purchased from Boehringer Mannheim. Reduced nicotinamide adenine dinucleotide (NADH), d-phenylalanine (Phe), potassium chloride, sodium chloride, and phenylmethylsulfonyl fluoride were all obtained from Sigma Biochemicals. Mono- and dibasic potassium phosphate were purchased from Fisher. [35S]dATP was purchased from Amersham Pharmacia Biotech. Oligonucleotides were purchased from Genosys Biotechnologies, Inc.

Site-directed Mutagenesis—The mutagenic oligonucleotide used for constructing the specific point mutant is as follows.

Mutant T340M: AGCCC CGCCC CATGC GGC 3′

The underlined sequence is the mutagenic nucleotide with the corresponding wild type sequence labeled below.

Mutagenesis of the PK gene was performed directly with the double-stranded plasmid pRK-PK (13) using the published procedure of Cheng et al. (5). Briefly, two oligonucleotide primers, a mutagenic primer and a selection primer, were simultaneously annealed to one strand of the denatured pRK-PK. The mutagenic primer, containing the sequence shown above, was designed to introduce the desired site-specific mutation into the rRKPK gene, whereas the selection primer with the nucleotide sequence of 5′-TTTCA CACCG CAGCT GGTGC ACTCT C-3′ was to mutate a unique Ndel restriction site in the pKK223–3 vector. The complementary strand, which incorporated both primers, was then synthesized by T4-DNA polymerase and circularized by T4-DNA ligase. Plasmids, propagated in a repair-deficient Escherichia coli strain Blh JM105 cells, containing the plasmid pRK-PK, with the T340M mutation were grown overnight in M9 minimal medium containing 100 µg/ml ampicillin and were inoculated 1/100 (v/v) into NZCYM medium containing 100 µg/ml ampicillin. Culture was grown at 37 °C until the optical density at 600 nm reached 0.7–1.0, at which point expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 120 µg/ml. After 12 h of induction, the cells were harvested by centrifugation in a Sorvall RC-5C centrifuge using the GS-3 rotor at 6000 rpm for 20 min.

Protein Purification and Preparation—All purification steps were performed at 4 °C except the SP-Sepharose chromatography, which was performed at room temperature using the published procedure of Cheng et al. (5) with the following minor modifications: 1) the final concentration of polyethylenimine was 0.35%, and 2) all buffer solutions contained 1 mM DTT and 0.4 mM FBP, the addition of which stabilizes the enzyme and ensures reproducible chromatographic behavior. Oxidation and the absence of FBP affect the oligomeric state of the enzyme, resulting in longer retention times on both chromatographic columns. Buffer A was 20 mM Tris-base, 100 mM KCl, 15% (v/v) glycerol, 1 mM DTT, 0.4 mM FBP, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.9. Buffer B was 5 mM KP, 1 mM EDTA, 1 mM DTT, 0.4 mM FBP, 5 mM MgSO4, 100 mM KCl, pH 7.5. Buffer C was the same as buffer B with no KCl and at pH 6.0.

Recombinant RPK was desalted before all experiments by filtration using a Superdex-200 AG on a FPLC system. The ammonium sulfate precipitate was concentrated by centrifugation at 14,000 rpm in an Eppendorf tabletop centrifuge for 5 min, and the pellet was resuspended in up to 300 µl of the appropriate buffer before loading onto the filtration column. Protein concentration was determined by absorbance

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at 280 nm, using an absorbivity of 0.54 ml/(mg cm) (15).

**Enzyme Kinetics**—The enzymatic activity of T340M rRKPK was determined by the lactate dehydrogenase-coupled enzyme assay (16). The reaction occurs in a solution consisting of 50 mM Tris base, 72 mM KCl, 7.2 mM MgSO₄, 0.5 mM NADH, 10 μg/ml lactate dehydrogenase, and varying amounts of Phe and FBP as indicated. The final concentration of ADP or PEP in the assay mixture was fixed at 2 or 20 mM, respectively, while varying the concentration of the other substrate. After adjusting the pH of the assay mix to 7.5 at 23 °C, lactate dehydrogenase that was equilibrated with TKM buffer (50 mM Tris, 72 mM KCl, 7.2 mM MgSO₄, pH 7.5) was added and the assay mix was finally brought to the desired volume. The reaction was started by the addition of 2–4 μl of PK to 1 ml of assay solution that had been equilibrated at 23 °C. The amount of PK present in the assay was about 0.5 μg. The decrease in absorption at 340 nm was followed as a function of time with a Hitachi U-2000 spectrophotometer to obtain v, the observed steady-state kinetic velocity. All data sets were fitted to the modified version of Hill equation as shown in Equation 1 (17).

\[ V = \frac{V_{\max} [S]}{K_m + [S]} \]  

(Eq. 1)

\( V_{\max} \) is the maximal velocity of each data set, \( [S] \) is the concentration of the variable substrate, \( n \) is the Hill coefficient, and \( K_m \) is a complex steady-state kinetic equilibrium constant that is equivalent to the \( K_m \) in the Michaelis-Menten equation, where \( n = 1 \).

**SDS-Gel Electrophoresis**—SDS-10% polyacrylamide gel electrophoresis was performed according to the method of Laemmli (18), followed by staining with Coomassie Blue. The markers used for molecular mass determination were: phosphorylase b (97 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa).

**Analytical Gel Chromatography**—For the analytical gel chromatography experiments, a Superdex-200 HR 10/30 column and FPLC equipment (Amersham Pharmacia Biotech, Uppsala, Sweden) were employed. The column was equilibrated with TRMD buffer (50 mM Tris, 72 mM KCl, 7.2 mM MgSO₄, 0.2 mM DTT, pH 7.5) at room temperature, with or without a ligand, and 100 μg of rRKPK samples were loaded onto the column. Fractions of 0.25 ml were collected, and PK activity was measured using the method described above. The following proteins were used for column calibration: catalase (240 kDa), aldolase (158 kDa), bovine serum albumin (66.2 kDa), calibration proteins II for gel chromatography; Combithek, Boehringer Mannheim).

**Circular Dichroism**—Far-UV CD spectra of T340M rRKPK and wild type rRKPK were measured with an Aviv 62 DS circular dichroism spectrometer. rRKPK solutions of 0.6 mg/ml and T340M rRKPK of 1.0 mg/ml were measured in the region from 190 nm to 260 nm with an Aviv 62 DS circular dichroism spectrometer. rRKPK solutions of 0.6 mg/ml and T340M rRKPK of 1.0 mg/ml were measured in the region from 190 nm to 260 nm with a 0.5-nm increment and 1-s interval. For each sample, three spectra were collected in the continuous mode with a radial stepsize of 0.003 cm, at 230 nm for rRKPK concentrations of 2.0 mg/ml lactate dehydrogenase, and 0.025 mg/ml and 0.050 mg/ml, and all other data were collected at 280 nm.

**Velocity Sedimentation**—Velocity sedimentation experiments were performed in a Beckman Optima XL-A analytical ultracentrifuge (Palo Alto, CA) with an An-60Ti rotor, equipped with absorption optics. The experiments were carried out at 30,000 rpm and 23 °C. Data were collected in the continuous mode with a radial stepsize of 0.003 cm, at 8-min intervals. Data were collected, on 400-μl samples loaded into double sector centerpieces, at 230 nm for rRKPK concentrations of 0.025 mg/ml and 0.050 mg/ml, and all other data were collected at 280 nm. Weight-average sedimentation coefficients were obtained by employing the Beckman data analysis software.

**RESULTS**

**Purification of T340M rRKPK**—The T340M rRKPK was purified in order to assess the kinetic and physical properties, employing the protocol established for rRKPK and rRMPK (5, 13). The purity of the recombinant T340M rRKPK was approximately 95% homogeneous based on a densitometric analysis of the SDS-polyacrylamide gel. Typically, 1 liter of culture results in 3 mg of purified T340M rRKPK, which is 2 times the yield of wild type rRKPK. Similar to the wild type rRKPK, the activity of T340M rRKPK is unstable. It is essential to carry out the purification method quickly. Generally, the sample should be dialyzing against buffer C within 10 h after cell disruption.

**Kinetic Characterization of T340M rRKPK**—Steady state kinetics obtained for both the wild type and T340M rRKPK, as a function of effectors (data not shown for wild type), with PEP as a variable substrate are shown in Fig. 2. Parameters derived from these steady state kinetics are summarized in Table I. In the absence of effectors, the saturation curves of the wild type and T340M rRKPK are significantly different. The value for \( K_{app,PEP} \) has increased from 0.16 mM for the wild type to 5.8 mM for the T340M rRKPK mutant, a more than 30-fold increase. The apparent cooperativity, quantitated by the Hill coefficient (n), was not significantly affected by the mutation. Recent reports showed that the interactions of PEP and Mg²⁺ with PK is coupled (19, 20). In view of the significant change in the value of \( K_{app,PEP} \), the effect of Mg²⁺ concentration was studied by monitoring PK activity at fixed concentrations of 2 mM ADP and 20 mM PEP and varying concentrations of MgSO₄. Activity of T340M rRKPK reached its maximum value at about 6 mM MgSO₄ after which there was no observable increase in activity with increasing concentration of MgSO₄. Thus, it can be concluded that the kinetic measurements were conducted in the presence of saturating concentration of MgSO₄.

The presence of the inhibitor Phe (2 mM) led to an increase in \( K_{app,PEP} \) for both wild type and T340M rRKPK. The increase in values for \( K_{app,PEP} \) is 19- and 4-fold in wild type and T340M mutant, respectively. There is no significant difference in the apparent cooperativity between wild type and T340M rRKPK in the presence of Phe. The presence of both Phe (2 mM) and the activator FBP (10 μM) decreases the apparent cooperativity and the value for \( K_{app,PEP} \) for both wild type and T340M rRKPK. For the wild type enzyme, the inhibitory effect of Phe is more than reversed by 10 μM FBP, indicated by a smaller \( K_{app,PEP} \) and Hill coefficient as compared with wild type in the absence of effectors. In the T340M rRKPK mutant, however, the presence of 10 μM FBP only partially reverses the inhibitory effect of Phe. The T340M mutation decreases \( k_{cat} \) by at least 2-fold under all three conditions tested. The effect of FBP alone was tested. The activity of T340M rRKPK increased with increasing concentration of FBP, as shown in Fig. 3. The normalized data were fitted to Equation 1. Values of 50 μM and 1.3 were obtained for \( K_{app,FBP} \) and \( n \), respectively. Maximum stimulation of activity was apparently achieved by 100 μM FBP. Effect of FBP on \( K_{app,PEP} \) was tested at two ligand concentrations of 100 and 400 μM. Results are shown in Fig. 2 and Table I. The values of \( K_{app,PEP} \) and \( k_{cat} \) remain at approximately 1.8 mM and 100 s⁻¹, respectively, despite a 4-fold increase in FBP concentration. These results imply that under these experimental conditions this mutant is operating at the maximum velocity of which this enzyme is capable.
Steady state kinetics of wild type and T340M rRKPK as a function of ADP concentration are shown in Fig. 4. The fixed concentration of PEP was 20 mM and 2 mM for experiments pertaining to the T340M mutant and the wild type enzyme, respectively. Parameters derived from these measurements are summarized in Table II. The $k_{cat}$ determined with ADP as a variable substrate is lower than that in the PEP titration (Table I). This is probably because at 20 mM PEP one has not reached substrate saturation for T340M rRKPK. These data indicate that the T340M mutation does not significantly affect the apparent kinetic parameters with respect to ADP as a substrate. A combination of all the steady state kinetic data implies that the T340M mutation apparently renders the enzyme less active with significantly lower affinity for PEP without perturbing ADP binding.

Circular Dichroism—The secondary structure of T340M rRKPK and wild type rRKPK were monitored by CD. There is no observable difference in molar ellipticity under the experimental conditions as shown in Fig. 5. The identity of the CD spectra for these two proteins indicates that the T340M mutant is folded into a similar motif as the wild type enzyme as determined by this spectroscopic technique.

Sedimentation Velocity—Wild type rRKPK undergoes self-association with a stoichiometry of 2D $\rightleftharpoons$ T (13).2 The association-dissociation occurs at the 1,2 subunit interface (Fig. 1). This is based on the fact that the sequence difference between the rMPK, which is a stable tetramer, and the rRKPK, which undergoes self-association, is at the 1,2 subunit interface. Moreover, the change in accessible surface area upon subunit assembly at the 1,2 subunit interface is about half of that of the 1,4 subunit interface. Thus, the expected energetic of interfacial interaction along the 1,2 subunit interface should be substantially less than that of the 1,4 interface. However, the T340M mutation is located at the 1,4 subunit interface, and an effect on the energetics at the 1,4 subunit interface might be observed. A weakening in the energetics along the 1,4 subunit interface is suggested by the sedimentation velocity results shown in Fig. 1. The identity of the CD spectra for these two proteins indicates that the T340M mutant is folded into a similar motif as the wild type enzyme as determined by this spectroscopic technique.

| rRKPK   | Effectors | $K_{app,PEP}$ | n | $k_{cat}$ |
|---------|-----------|---------------|---|----------|
|         |           | $\text{mM}$  |   | $s^{-1}$ |
| WT      | None      | 0.16 ± 0.005  | 2.1 ± 0.2 | 200 ± 2  |
| WT      | I         | 3.0 ± 0.2     | 2.3 ± 0.2 | 192 ± 9  |
| WT      | A and I   | 0.08 ± 0.005  | 1.5 ± 0.1 | 191 ± 3  |
| T340M   | None      | 5.8 ± 0.2     | 1.9 ± 0.1 | 102 ± 2  |
| T340M   | I         | 25.8 ± 1.2    | 2.3 ± 0.2 | 59 ± 2   |
| T340M   | A and I   | 12.8 ± 1.8    | 1.3 ± 0.2 | 78 ± 5   |
| T340M   | AA        | 1.89 ± 0.08   | 2.0 ± 0.1 | 97 ± 2   |
| T340M   | AAA       | 1.83 ± 0.09   | 1.7 ± 0.1 | 101 ± 2  |

*Values calculated assuming the presence of saturating PEP (20 mM). The values after the "±" sign are the errors obtained upon fitting a particular saturation curve.

**Fig. 3.** FBP activation of T340M rRKPK. The activity was monitored at fixed concentrations of 2 mM ADP and 5 mM PEP in the presence of varying concentrations of FBP. The enzyme activity in the absence of FBP was used as the reference. The ordinate is in the unit of $(V_{FBP}/V_o - 1)$ where $V_{FBP}$ and $V_o$ are the activities in the presence and absence of FBP, respectively.

**Fig. 4.** Kinetic properties of the wild type rRKPK (Δ), and the T340M rRKPK (●). ADP was the variable substrate in the presence of fixed 2 mM and 20 mM PEP for wild type and T340M rRKPK, respectively. The lines through the data sets are the results of nonlinear fitting to Equation 1. The kinetic parameters resulting from the fittings are shown in Table II. The ordinate is shown in nanomoles of product/min.

**Fig. 5.** CD spectra in the far-UV region of wild type rRKPK and T340M rRKPK in TKMD at 23 °C as a function of protein concentration. Figure shows results for wild type rRKPK at 0.6 mg/ml (Δ) and T340M rRKPK at 1.0 mg/ml (●).
interface might change the stoichiometry of the self-association reaction from dimerization of dimers to tetramerization of monomers. The stoichiometry of the self-association reaction can be evaluated by applying the Gilbert theory to the sedimentation data (21). To describe the self-association of T340M rRKPK quantitatively, the ability to aggregate was monitored by sedimentation velocity. Weight-average sedimentation coefficients of rRKPK were determined within the protein concentration range of 0.025–1.5 mg/ml at pH 7.5 and 23 °C in TKMD buffer. As expected for a system undergoing self-association, the weight-average sedimentation coefficient is a function of protein concentration, as depicted in Fig. 6. At higher protein concentration, the weight-average sedimentation coefficient converges to a value greater than 10 S. A similar value was obtained for the tetrameric rRKPK and rRKPK (5, 13).2 Extrapolation of a double reciprocal plot of the weight-average sedimentation coefficient versus concentration to infinite concentration yielded a value of 10.50 S for the largest aggregate of this T340M mutant. The absorption optics of the analytical ultracentrifuge places the lower limit of PK that can be monitored at 2.5 μg/ml, as shown in Fig. 6. Further analysis of the sedimentation data was conducted to define the stoichiometry and equilibrium constants of the self-association reaction because weight average sedimentation coefficient is defined by

$$\tilde{s} = \frac{s}{C} \sum C_i$$  \hfill (Eq. 2)

$s_i^0$ and $C_i$ are the intrinsic sedimentation coefficient and concentration of the $i$th species, respectively. The relationship between $\tilde{s}$ and total protein concentration is a function of stoichiometry and equilibrium constants and is related by Equation 3.

$$\tilde{s} = \frac{s}{(1 - g_i C_i K_i) \sum K_i C_i}$$  \hfill (Eq. 3)

g$_i$ is the hydrodynamic non-ideality term, $K_i$ is the association constant governing the formation of $i$-mer from monomer and $K_i = 1$, by definition, and $C_i$ is the monomer concentration. Fitting the data of $\tilde{s}$ versus $C$ requires an estimation of values of $s_i^0$. The $s_{20,w}$ values of the monomer and dimer can be calculated employing Equation 4.

$$s_i^0 = s_i^w (1 - g_i)$$  \hfill (Eq. 4)

The calculated sedimentation coefficients, based on a tetramer of 10.5 S, are 4.16 S and 6.61 S for the monomer and dimer, respectively. This relationship assumes spherical symmetry for all species (22, 23).

The concentration dependence of the weight-average sedimentation coefficient was fitted to different modes of association using the calculated $s_{20,w}$ values for the monomer and dimer, and the experimentally determined $s_{20,w}$ value of the tetramer. The results of the least-squares fittings are described in Table III. The calculated data employing model I and II fit equally well to the experimental data. Fitting to model III, however, results in non-random residuals, which excludes model III as a possible model. Expressing the reaction boundary of a system in rapid dynamic equilibrium as weight-average sedimentation coefficient can result in the loss of valuable information. The shape of the sedimenting boundary can be very diagnostic for detecting the mode of association according to the Gilbert theory, which predicts a single sedimenting peak for macromolecular dimerization ($n = 2$) and resolution into bimodal sedimentation profiles for higher order polymerization reactions ($n \geq 3$). The appearance of bimodality in the higher-order polymerization reaction is a function of the association constants and total macromolecular concentration in the plateau region.

Comparing the simulated and experimental sedimentation patterns can provide diagnostic information in support of a model describing a self-association equilibrium (21). Sedimentation velocity profiles were simulated based on a method developed by Cox (24) and adopted by this laboratory in earlier studies (25–27). The simulated profiles describe a derivative of a sedimentation boundary at a fixed sedimentation time and solute concentration, employing the fitted association constant and values for $s_{20,w}$ for models I and II. Model I fits the $s_{20,w}$ versus $C$ data in Fig. 6 to a monomer-tetramer mode of association. The simulated sedimentation profiles for two different protein loading concentrations are shown in Fig. 7A. Because the stoichiometry of polymer formation is 4 ($n = 4$), the observed bimodality in Fig. 7A is consistent with the Gilbert theory. Model II fits the $s_{20,w}$ versus $C$ data in Fig. 6 to a dimer-tetramer mode of association. The dimerization constant derived from data analysis of Fig. 6 and $s_{20,w}$ values of the dimer and tetramer were used to simulate a sedimentation pattern for two different protein loading concentrations (Fig. 7B). Each of the simulated sedimentation pattern exhibits a single sedimenting peak as predicted by the Gilbert theory for a macromolecular dimerization ($n = 2$).

Derivatives of experimental sedimentation patterns, at the protein loading concentrations and sedimentation times used in the simulations, are plotted in Fig. 7C. It is evident, based on the shape, dependence on protein loading concentration, and amplitude on the ordinate, that the experimental data resemble Fig. 7B best. Therefore, it can be concluded that the mode of association is not affected by the T340M mutation and remains as a dimerization of dimers to tetramer. The fitted equilibrium constant for this mutant is 220 ± 20 ml/mg as compared with 28 ± 3 ml/mg determined for the wild type rRKPK.

Analytical Gel Chromatography—In an earlier report, analytical gel chromatography was employed to show that wild type rRKPK undergoes self-association and that the presence of metabolites significantly affects the equilibrium (13).2 The same approach was used to monitor changes in molecular size of T340M rRKPK as a function of protein concentration and
Phe concentration.

The elution profile of T340M rRKPK at 50 µg/ml, as shown in Fig. 8, is identical to that of tetrameric rRKPK and rRMPK (data not shown), with respect to both elution volume and shape of the profile (13). This indicates that T340M rRKPK is predominantly tetrameric at this concentration, which is about 10-fold higher than the dissociation constant for the dimer-tetramer reaction. The elution volume of wild type rRKPK at 12 µg/ml implies the presence of a molecule substantially less than tetrameric PK. In accordance to a dimerization constant of 28 ml/mg (13), then rRKPK should exist predominantly as dimer at this protein concentration. In contrast, at 2.5 mM Phe (data not shown), indicates that the mutant remains equilibrium to the dimeric form. The elution profile of T340M rRKPK at 50 µg/ml, as shown in Fig. 8, is identical to that of tetrameric rRKPK and rRMPK (Fig. 8). This observation implies that the T340M mutant exhibits a higher propensity to self-association, a conclusion in complete agreement with the sedimentation data. The self-association of wild type rRKPK is linked to the binding of Phe, such that Phe binding shifts the equilibrium to the dimeric form. The elution profile of T340M rRKPK at 10 µg/ml in the presence of 2 mM Phe (Fig. 8) or 12 mM Phe (data not shown), indicates that the mutant remains predominantly tetrameric. The lack of response to Phe in the analytical gel chromatography experiments is not the result of a decreased affinity of T340M rRKPK for Phe, inasmuch as the kinetic properties are responsive to 2 mM Phe (Fig. 2). Therefore, it can be concluded from these experiments that the T340M mutation at the 1,4 subunit interface results in significant enhancement in the energetics of subunit assembly at the 1,2 subunit interface.

**DISCUSSION**

The major observations of this study are that T340M mutation exerts a dominant effect on mammalian PK by lowering the catalytic efficiency and apparent affinity for PEP, and a decrease in responsiveness to the presence of effectors while exhibiting no apparent effect on ADP binding. Furthermore, the effects of T340M mutation are propagated through long range communication to the active site approximately 20 Å away. This conclusion is supported by the observation that there is communication between the active site and both subunit interfaces. Mutation of this highly conserved threonine to methionine in rRKPK (this study), rRMPK (5), and human erythrocyte and liver PK isozymes (7–9) results in a similar observation of decrease in enzyme activity. These results provide support for the hypothesis that the molecular mechanism of regulation in PK is conserved. The verification of this assumption significantly increases the efficiency of selecting functionally important structural elements. The information embedded in human erythrocyte PK variants can be revealed by studying the equivalent mutants of the other isozymic forms of PK. The sites of mutation and the nature of amino acid substitution reported in these human genetic variants can serve as leads in probing the molecular mechanism of allosteric regulation in mammalian PK.

The T340M mutation significantly alters the kinetic properties of the enzyme. By comparing the kinetic behavior of the wild type and T340M rRKPK, there is an approximately 50% decrease in the value of $k_{cat}$. The effect of mutation on the steady state affinity for PEP is based on the observed perturbation on the value of $K_{app,PEP}$. Although one may question the validity in equating these kinetically determined $K_{app}$ parameters to actual equilibrium binding constants in rRKPK, it was shown by independently determined ligand binding constants that the $K_{app}$ parameters are reasonable approximation of binding constants in RMPK (26–29). It is most interesting to us that the same effects of T340M mutation were observed on the kinetic behavior of the other form of PK, the rRMPK isozyme (5). Thus, the sequence differences between the two isozymes along the 1,2 subunit interface do not alter the dominating, negative effects of the T340M mutation. These effects are a decrease in catalytic efficiency, decrease in apparent affinity for PEP, and decreased responsiveness to allosteric effectors.

An important feature of allosteric regulation is the communication between ligand binding sites and subunits of an oligomeric enzyme such as PK. An outstanding advantage of rRKPK to elucidate the communication network is its propensity to undergo a dynamic association-dissociation reaction. The change in the energetics of subunit assembly can be employed to reflect a communication via this subunit interface (13). In wild type rRKPK, it was shown that binding of all metabolites communicates through the 1,2 subunit interface. From the results of both analytical gel chromatography and sedimentation velocity experiments, it was apparent that the T340M mutation shifts the equilibrium in favor of the tetrameric form. Thus, an obvious conclusion is that there is communication between residue 340 and the 1,2 subunit interface, although the exact mechanism of communication has to be defined. The mechanism could involve a direct pathway between these two sites, or it might be indirect through a global structural perturbation by the T340M mutation. It is interesting to note that analogous to the observed correlation between subunit assembly and metabolite binding, a possible linkage can be considered between the self-association equilibrium and the effect of Phe on the kinetic behavior of the T340M mutant. In wild type rRKPK, the kinetic behavior is more responsive to Phe and the presence of this ligand significantly shifts the subunit assembly equilibrium toward the dimeric form. However, in the presence of up to 12 mM Phe, the T340M rRKPK remains predominantly tetrameric. These results indicate that the T340M mutation in rRKPK affects the intrinsic properties of the 1,2 subunit interface, and there is an apparent correlation between the decrease in responsiveness to Phe in enzyme kinetics and subunit assembly equilibrium, namely less response with a decreased ability to shift the subunit assembly equilibrium. Residue 340 not only communicates with the 1,2 subunit interface, although the exact mechanism of communication has to be defined. The mechanism could involve a direct pathway between these two sites, or it might be indirect through a global structural perturbation by the T340M mutation. It is interesting to note that analogous to the observed correlation between subunit assembly and metabolite binding, a possible linkage can be considered between the self-association equilibrium and the effect of Phe on the kinetic behavior of the T340M mutant. In wild type rRKPK, the kinetic behavior is more responsive to Phe and the presence of this ligand significantly shifts the subunit assembly equilibrium toward the dimeric form. However, in the presence of up to 12 mM Phe, the T340M rRKPK remains predominantly tetrameric. These results indicate that the T340M mutation in rRKPK affects the intrinsic properties of the 1,2 subunit interface, and there is an apparent correlation between the decrease in responsiveness to Phe in enzyme kinetics and subunit assembly equilibrium, namely less response with a decreased ability to shift the subunit assembly equilibrium. Residue 340 not only communicates with the 1,2 subunit interface, there is also communication between residue 340 and the active site approximately 20 Å away. This conclusion is supported by the observation that the catalytic efficiency of the T340M mutant is significantly decreased. Communication between the active site and the 1,2 subunit interface was estab-
Studies in this report identify the communication between the T340M mutation at the 1,4 subunit interface and the 1,2 subunit interface. This completes a triangulation of communication network within the rRKPK subunit between the T340M mutation at the 1,4 subunit interface, the 1,2 subunit interface, and the active site. This is the first report that identifies the communication between these functionally important structural elements in PK and in turn addresses a fundamental issue in elucidating the regulatory mechanism of PK by identifying the network of communication between functionally important sites, such as ligand binding sites and subunit interfaces.

Results from small angle neutron scattering experiments and computation modeling led to a proposal that the conversion of inactive RMPK to the active state is associated with a rotation of the B domain toward the A domain along the 1,4 subunit interface (31). As a consequence of this domain rotation, one of the new contact sites involves residue 340. Thus, the observed perturbation of kinetics and communication pathway reported in this study is consistent with the earlier proposal. Being encouraged by these consistent results, it is useful to examine the structure of RMPK determined by Larsen et al. (11) in order to develop a model for the transmission of information between the 1,4 subunit interface and the active site.

The model indicates that the closed cleft conformation of the active state is stabilized by intersubunit electrostatic interactions between Asp-177 in the B domain of subunit 1 with Arg-341 of the A domain in the subunit across the 1,4 subunit interface. Stabilization of the active state by an electrostatic interaction between Arg-341 and Asp-177 is consistent with results of a study of RMPK that indicated a loss of activity in the presence of high salt concentrations (32). Arg-341 is part of a helix 12, which includes residues 341–353, that constitutes part of the 1,4 subunit interface. It is possible that a minor change, such as a T340M mutation, at the 1,4 subunit interface is propagated through helix 12, resulting in a perturbation of the proper positioning of its neighboring Arg-341 with Asp-177, resulting in the destabilization of the active state. Asp-177, Arg-341, and Thr-340 are conserved residues in all known PK sequences (10). This model is consistent with the kinetic observations of the T340M mutants in rRKPK, rRMPK, and human erythrocyte PK (5, 7–9) that the T340M mutation in all three PK isozymes lead to the same net result, namely significant deficiency in PK activity. Of course, the validity of this proposed model awaits the high resolution structural information of this mutant and further experimental results of mutants and solution biophysical characterizations of these mutants. Nevertheless, it is interesting to note that Mattevi et al. (33) identified the equivalent of residue 341 in E. coli PK as playing a role in intersubunit salt bridge formation to stabilize the T-state enzyme. Alternatively, a communication pathway between residue 340 and active site may involve transmission of information between residue 340 and the 1,2 subunit interface,
which in turn communicates to the active site. A communication between the 1,2 subunit interface and active site has been demonstrated in an earlier study (13). These two models can rationalize part of the communication network that couple different locations within the PK tetramer.

The significance of this mutation in affecting the molecular mechanism of regulation has yet to be determined, inasmuch as allosteric regulation is the net consequence of linked functions in which the binding affinity of one ligand is linked to that of another ligand and structural equilibria as formulated mathematically by Wyman (12). As a consequence of these linkages, the observed fitted constants are apparent parameters and can be distinct from their intrinsic values. To assess the actual effect of the T340M mutation on the regulatory mechanism at the molecular level, it is imperative to identify the intrinsic properties of the various linked equilibria that are affected. Further studies are being conducted to dissect these linked equilibria.3

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