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Functional Energetic Landscape in the Allosteric Regulation of Muscle Pyruvate Kinase II. Fluorescence Study

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Abstract

The energetic landscape of the allosteric regulatory mechanism of rabbit muscle pyruvate kinase (RMPK) was characterized by isothermal titration calorimetry (ITC). Four novel insights were uncovered 1. ADP exhibits a dual property. Depending on the temperature, ADP can regulate RMPK activity by switching the enzyme to either the R- or T-state. 2. The assumption that ligand binding to RMPK is state dependent is only correct for PEP but not Phe/ADP. 3. The pH effect on the regulatory behavior of RMPK is partly due to the complex pattern of proton release or absorption linked to the multiple linked equilibria which govern the activity of the enzyme. 4. The R $\leftrightarrow$ T equilibrium is accompanied by a significant ΔC_p rendering RMPK most sensitive to temperature under physiological conditions. In order to rigorously test the validity of conclusions derived from the ITC data, in this study a fluorescence approach, albeit indirect, that tracks continuous *structural* perturbations was employed. Intrinsic Trp fluorescence of RMPK in the absence and in the presence of substrates phosphoenolpyruvate (PEP) and ADP, and the allosteric inhibitor Phe was measured in the temperature range between 4°C and 45°C. For data analysis the fluorescence data were complemented by ITC experiments to obtain extended data set allowing more complete characterization of the RMPK regulatory mechanism. Twenty-one thermodynamic parameters were derived to define the network of linked interactions involved in regulating the allosteric behavior of RMPK through global analysis of the ITC and fluorescent data sets. *In this study 27 independent curves with more than 1600 experimental points were globally analyzed.* Consequently, the consensus results not only substantiate the conclusions derived from the ITC data but also structural information characterizing the transition between the active and the inactive state of RMPK and the antagonism between ADP and Phe binding. The latter observation reveals a novel role for ADP in the allosteric regulation of RMPK.

Keywords

Two state model; global analysis; allosterism; fluorescence; calorimetry

In the previous article of this series isothermal titration calorimetry, ITC, was employed to define the energy landscape of allosteric regulation of rabbit muscle pyruvate kinase, RMPK (1). Based on a simple two-state model for the cooperative binding of ligands by macromolecules earlier described by Monod and customized for the tetrameric RMPK by Oberfelder et al. (2), four novel insights on the mechanism of allosteric regulation of RMPK

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were uncovered: 1. A temperature dependent cross over of ADP affinity towards R and T-state; more favorably to the R- and T-state at high and low temperature, respectively. Thus, ADP does not only serve as a substrate but also plays an important and intricate role in regulating RMPK activity. 2. The binding of Phe is negatively coupled to that of ADP in addition to the shifting of the $R \leftrightarrow T$ equilibrium. Thus, the assumption that ligand binding to RMPK is state dependent is only correct for PEP but not for the Phe/ADP pair. 3. The release or absorption of protons linked to the various equilibria is specific to the particular reaction. As a consequence, pH will exert a complex effect on these linked equilibria with the net effect being manifested in the regulatory behavior of RMPK. 4. The $R \leftrightarrow T$ equilibrium is accompanied by a significant ΔC_p enabling the change of state of RMPK to be particularly sensitive at physiological conditions. While the approach of ITC is versatile and powerful, it is an indirect measurement that does not identify the specific interaction among the many linked reactions. Reactions with significant heat change may dominate the signal and obscure others. Thus, we wish to acquire data that are complementary to the ITC data. The complementary should provide signal from structural changes. In this study a fluorescence approach, albeit indirect, that tracks continuous structural perturbations was employed.

The choice of fluorescence is based on the fact that execution of biological function by proteins depends highly on an internal mobility encoded by their structures and sequences. These movements are inherent to the structure and to the interactions within the molecule (6). In fact, proteins even in their native states assume different conformations differing in structural details (7,8). Due to the high sensitivity of indole emission to local interactions and changes in the microenvironment induced by conformational changes, either local or involving the entire macromolecule, tryptophan residues are excellent intrinsic probes for fluorescent monitoring of protein structural transitions in solution (9,10). Thus, fluorescence of tryptophan residues is employed as the probe to track ligand binding and structural changes.

Analysis of X-ray data revealed that each RMPK subunit is composed of three major domains (11), as shown in Fig. 1. The B-domain is exposed to the solvent and is attached to the central A-domain by a flexible hinge region. Domains A and B form a cleft adjacent to the active site. Previous studies indicated that binding of metabolites induces changes in the hydrodynamic properties of RMPK (2,12–14). In particular, binding of PEP and metal ions required for activity causes the enzyme to assume a compact symmetric conformation (the R-state), whereas the allosteric inhibitor Phe converts RMPK to the inactive form with a significantly expended structure (the T-state). Since the secondary structure of the enzyme was shown to remain unaltered (15) significant domain movement have to take place during the conformational transitions from the active to the inactive state. Because each RMPK subunit contains three Trp residues that can serve as intrinsic probes these transitions can be followed by Trp fluorescence. Two Trps are located in the C-domain and Trp157 is located in the movable A-domain near the binding site (16). This location is very favorable for fluorescent monitoring of the ligand binding.

In this study environmental sensitivity of Trp fluorescence was employed for spectroscopic monitoring of the $R \leftrightarrow T$ equilibrium shifts induced by PEP, ADP, and Phe binding. Fluorescence data sets were simultaneously analyzed with the ITC data from the previous paper (1). This complex approach allowed an “over” determination of the model with multiple overlapping signal and to extract thermodynamic parameters that were inaccessible by analysis of single curves or the ITC data only.

MATERIALS AND METHODS

Materials

Samples preparations and material were identical to procedures described in detail in the previous paper (1).

Fluorescence

Fluorescence experiments were performed on the SLM 8000 spectrofluorometer in TKM buffer (at pH 7.5 contained 50 mM Trizma-base, 72 mM KCL, and 7.2 mM MgSO₄) at a protein concentration of 0.1 mg/ml. Temperature of the sample was measured in a cuvette by a thermocouple (BAT-12) with an accuracy of ± 0.15 °C.

Isothermal fluorescent titrations (IFT)

Ligands were added to the sample by an automatic burette (ABU80, Radiometer Copenhagen) remotely controlled by TTL signals from the fluorometer. Samples were gently mixed by a magnetic stirrer during the titration. In order to avoid inner filter effects caused by an addition of ligands and by protein dilution as well as an excitation of tyrosines, the excitation wavelength was set to 300 nm (1 nm slit width). Fluorescence of RMPK was collected at 350 nm where contribution of Raman scattering generated by the excitation light in the aqueous solvent was negligible. All titrations were repeated with buffer substituted for the sample and the background signal was collected. Raw fluorescence data were corrected for a dilution of protein and for a small background emission of buffer and ligands. Then the IFT curves were normalized by fluorescence intensity of the unliganded RMPK. The normalization factor was determined at the beginning of every titration. Dedicated computer program was developed for these automatic fluorescence titrations.

Fluorescence temperature scans (FTS)

Four samples were prepared and placed in a four-cell temperature-controlled cuvette holder in the spectrofluorometer. In particular, the first cuvette contained unliganded RMPK; the second cuvette contained the same RMPK concentration with a saturating concentration of a ligand; the third and the fourth cuvette contained buffer and buffer with the ligand, respectively. Then the corrected intensity ratio $I_{lig}/I_0 = (I_{(PK+lig)} - I_{(buf+lig)}) / (I_{PK} - I_{buf})$ was automatically measured as a function of temperature. Temperature was slowly increased by a programmable water bath (Neslab, RTE-110) at a rate of 0.2°C/min. This rate of increase was slow enough to guarantee that intensity readings from all four cuvettes were done at the same temperature. Temperature of the sample was measured directly in the cuvette at the very moment of the data acquisition using a thermocouple thermometer (Physitemp, BAT-12) connected to the data acquisition board (DAP 1200e/6). Temperature readings and data logging was synchronized by TTL signals with the spectrofluorometer. A dedicated computer program was developed for the instrumental control. The tryptophan emission was isolated by a combination of a UG1 absorption filter and a long-pass filter with a cutoff wavelength of 345 nm. In order to eliminate a temperature-induced pH shift of the TKM buffer, samples were changed every 5°C and the buffer with pH corrected for the temperature change was used. Therefore the sample pH never differed more than ± 0.06 pH units from the preset value of 7.50. Reversibility of the observed fluorescence changes was monitored by a repetition of the heating-cooling cycle with the same sample. Data from repeated measurements overlapped with the original ones. The complete titration curves were constructed by overlapping the data from adjacent temperature intervals.

Global fitting

Global fitting (17) is a powerful method for discerning between models and recovery of model parameters. It has been described in detail elsewhere (17,18). The method is based on an

ordinary non-linear least square fitting (19) and it allows for simultaneous analysis of multiple data sets acquired under different conditions and with different techniques. During the global fitting the overall sum of weighted squared deviations of calculated values from the measured data (χ^2) is minimized in order to obtain model parameters that are consistent with all data sets. A home-coded program utilizing the Marquardt-Levenberg minimization procedure (20) was employed to simultaneously analyze both the fluorescence and the previously published ITC data (1). The model described in the previous paper (1) and extended in this paper encompassed all the experimental data by a complex linkage of thermodynamic parameters between different data sets. As a consequence, many model parameters were inherently common for several different data sets. Such an over determination helped validate the model and recover the model parameters with a resolving power unequaled by a conventional data analysis (21–27).

Model description

Our model is based on a two-state model for cooperative binding of ligands to macromolecules that has been published by Monod et al. (28) and adapted by Oberfelder et al. (2) for the tetrameric RMPK. Description and application of this model for analysis of calorimetric RMPK data can be found in the previous paper (1). Briefly, experimental data suggest (1–4,12) that unliganded RMPK assumes two major conformations that are in equilibrium, $R \leftrightarrow T$. Enzyme in the R-state exhibits high activity; T is an inactive state. The equilibrium constant between the two unliganded states is:

$$L_0 = [T_0]/[R_0] \quad (1)$$

and a temperature dependence of L_0 is given by equation:

$$-RT \cdot \ln(L_0) = \Delta H_{R \leftrightarrow T} - T \cdot \Delta S_{R \leftrightarrow T} \quad (2)$$

where $\Delta H_{R \rightarrow T}$ and $\Delta S_{R \rightarrow T}$ is enthalpy and entropy change, respectively, associated with the $R \rightarrow T$ transition. Calorimetric data has shown that an isobaric heat capacity change is associated with the transition, therefore:

$$\Delta H_{R \leftrightarrow T} = \Delta H_{0,R \leftrightarrow T} + \Delta C_{p,R \leftrightarrow T} \cdot (T - T_0) \quad (3)$$

and

$$\Delta S_{R \leftrightarrow T} = \Delta S_{0,R \leftrightarrow T} + \Delta C_{p,R \leftrightarrow T} \cdot \ln(T/T_0) \quad (4)$$

In the presence of a single ligand the ligand binds to both states and the reaction scheme changes to:

This is a multiple equilibria system where dissociation constants $K_{\text{lig}}^{\text{state}}$ of the ligand *lig* to the R and T states are temperature dependent:

$$RT \cdot \ln(K_{\text{lig}}^{\text{state}}) = \Delta H_{\text{lig}}^{\text{state}} - T \cdot \Delta S_{\text{lig}}^{\text{state}} \quad (5)$$

In order to quantitatively describe fluorescence changes induced by separate or simultaneous binding of one substrate (ADP or PEP) and the inhibitor (Phe) to RMPK, it was assumed that each multiply liganded R-state (R_{ij}) and T-state (T_{ij}) has a unique fluorescence quantum yield characterized by a fluorescent coefficient a_{ij} and b_{ij} , respectively. The subscript *i* and *j* denotes a number of substrate and inhibitor molecules, respectively, bound to the RMPK tetramer. Since each RMPK subunit has a unique binding site for each substrate and for the inhibitor as well, both subscripts assume values from 0 to 4. For example, $[R_{23}]$ is the concentration of the R-state with 2 molecules of one of the substrates and 3 molecules of inhibitor bound; $[T_{00}]$ is the concentration of an unliganded RMPK tetramer in the T-state.

The ligation-dependent part of a_{ij} and b_{ij} is a minor, perturbation on the level of a subunit that contributes to a small extent in addition to the major, ligation-independent, change of the fluorescent coefficients caused by the concerted $R \leftrightarrow T$ transition. The binding-dependent modulation of the emission quantum yield may have multiple origins. Since the intramolecular quenching does not necessarily need an actual collision of the groups, the quantum yield can be influenced by long-range through-space interactions, e.g. by a binding-induced change of the local electrostatic field (43). Intensity variations can be also coupled to changes of the subunit internal dynamics that can change upon the ligand binding (44).

The measured emission intensity can be calculated as a simple sum of intensities over all possible ligation states:

$$I = C \cdot \frac{\sum_{i,j=0}^4 (a_{ij}[R_{ij}] + b_{ij}[T_{ij}])}{\sum_{i,j=0}^4 ([R_{ij}] + [T_{ij}])} \quad (6)$$

where *C* is a proportionality constant including quantum yield and instrumental parameters. Intensity I_0 of the unliganded state is therefore:

$$I_0 = C \cdot \frac{a_{00} + L_0 \cdot b_{00}}{1 + L_0} \quad (7)$$

where $L_0 = [T_{00}]/[R_{00}]$ is the equilibrium constant for the $R \leftrightarrow T$ equilibrium.

R_{ij} and T_{ij} can be expressed as (29):

$$R_{ij} = R_{00} \binom{4}{i} \binom{4}{j} \left(\frac{[\text{Sub}]}{K_{\text{Sub}}^R} \right)^i \left(\frac{[\text{Inh}]}{K_{\text{Inh}}^R} \right)^j \quad (8)$$

$$T_{ij}=T_{00}\binom{4}{i}\binom{4}{j}\left(\frac{[\text{Sub}]}{K_{\text{Sub}}^T}\right)^i\left(\frac{[\text{Inh}]}{K_{\text{Inh}}^T}\right)^j \quad (9)$$

where [Sub] and [Inh] are concentrations of a chosen substrate and the inhibitor, respectively, and $K_{\text{Sub}}^{\text{state}}$ and $K_{\text{Inh}}^{\text{state}}$ are dissociation constants from the R or T-state of RMPK. After substitution of eq. (8), and eq. (9) into eq. (6) and normalization of the result by I_0 , Eq.(7), then

$$\frac{I}{I_0} = \frac{1+L_0}{1+\tilde{b}_{00}L_0} \cdot \frac{\sum_{i,j=0}^4 \binom{4}{i}\binom{4}{j} [\text{Sub}]^i [\text{Inh}]^j \left[\tilde{a}_{ij}(K_{\text{Sub}}^R)^{-i}(K_{\text{Inh}}^R)^{-j} + \tilde{b}_{ij}L_0(K_{\text{Sub}}^T)^{-i}(K_{\text{Inh}}^T)^{-j} \right]}{\sum_{i,j=0}^4 \binom{4}{i}\binom{4}{j} [\text{Sub}]^i [\text{Inh}]^j \left[(K_{\text{Sub}}^R)^{-i}(K_{\text{Inh}}^R)^{-j} + L_0(K_{\text{Sub}}^T)^{-i}(K_{\text{Inh}}^T)^{-j} \right]} \quad (10)$$

Equation (10) is temperature dependent due to the temperature dependence of L_0 and all dissociation constants, eq.(2–5). Normalized fluorescence coefficients $\tilde{a}_{ij}=a_{ij}/a_{00}$ and $\tilde{b}_{ij}=b_{ij}/a_{00}$ characterize relative fluorescence changes upon binding of a ligand. The reference state is the unliganded R-state. For modeling of the $\tilde{a}_{ij}$ and $\tilde{b}_{ij}$ coefficients, we assumed that each binding event of the particular ligand causes the same fluorescence change. This seems to be a reasonable first-order approximation for homotetrameric RMPK where the binding-induced fluorescence change can be envisioned as a direct perturbation of the Trp microenvironment by the ligand on the level of the subunit. Then $\tilde{a}_{ij}$ and $\tilde{b}_{ij}$ can be calculated as:

$$\tilde{a}_{ij}=1+i \cdot \xi_{\text{Sub}}^R+j \cdot \xi_{\text{Inh}}^R; \tilde{b}_{ij}=\tilde{b}_{00}+i \cdot \xi_{\text{Sub}}^T+j \cdot \xi_{\text{Inh}}^T \quad (11)$$

The term $\tilde{b}_{00}$ represents relative fluorescent intensity of the unliganded T-state relative to the intensity of the unliganded R-state and reflects a structural difference between the two states. $\xi_{\text{Sub}}^{\text{state}}$ and $\xi_{\text{Inh}}^{\text{state}}$ are terms for fluorescence increments caused by a single binding event on the level of one RMPK subunit. Depending on the nature of perturbation, these increments can generally be either negative or positive. Equation (10) can be used to calculate I/I_0 for any concentration of a single substrate (ADP or PEP) in the presence of any fixed concentration of inhibitor.

Temperature dependence of the fluorescent intensity $^{\text{sat}}I_{\text{lig}}$ for RMPK *saturated* by a single ligand *lig* (this can be either substrate or inhibitor), relative to the fluorescent intensity of unliganded enzyme I_0 can be derived from eq. (10) using eq. (11). For high concentration of ligand (when RMPK is fully saturated) we obtain:

$$\frac{^{\text{sat}}I_{\text{lig}}}{I_0} = \lim_{[\text{lig}] \rightarrow \infty} \left(\frac{I}{I_0} \right) = \frac{1+L_0}{1+\tilde{b}_{00}L_0} \cdot \frac{(1+4\xi_{\text{lig}}^R)+L_0(K_{\text{lig}}^R/K_{\text{lig}}^T)^4(\tilde{b}_{00}+4\xi_{\text{lig}}^T)}{1+L_0(K_{\text{lig}}^R/K_{\text{lig}}^T)^4} \quad (12)$$

At a saturating PEP concentration and temperatures between 5 °C and 45 °C the RMPK molecule was shown to be fully in the R-state as a consequence of the preferential binding of PEP to the active state (1). Then the term $L_0(K_{\text{PEP}}^R/K_{\text{PEP}}^T)^4 \ll 1$ may be neglected and Eq. (12) is reduced to:

$$\frac{{}^{\text{sat}}I_{\text{PEP}}}{I_0} = \frac{(1+L_0)(1+4\xi_{\text{PEP}}^R)}{1+\tilde{b}_{00}L_0} \quad (13)$$

Similarly, at saturating Phe concentration the $R \leftrightarrow T$ equilibrium was in favor of the T-state by preferential binding of Phe to this state. As a consequence $L_0(K_{\text{Phe}}^R/K_{\text{Phe}}^T)^4 \gg 1$ and eq. (12) reduces to:

$$\frac{{}^{\text{sat}}I_{\text{phe}}}{I_0} = \frac{(1+L_0)(\tilde{b}_{00}+4\xi_{\text{phe}}^T)}{1+\tilde{b}_{00}L_0} \quad (14)$$

When eq. (14) is divided by eq. (13) we obtain a measurable quantity:

$$\frac{{}^{\text{sat}}I_{\text{phe}}/{}^{\text{sat}}I_{\text{PEP}}}{\tilde{b}_{00}+4\xi_{\text{phe}}^T} = \frac{1}{1+4\xi_{\text{PEP}}^R} \quad (15)$$

Importantly, eq. (15) shows that the ${}^{\text{sat}}I_{\text{phe}}/{}^{\text{sat}}I_{\text{PEP}}$ ratio is a function of the fluorescent coefficients only and, as a consequence, information about temperature dependence of these coefficients can be extracted from the experimentally measured ${}^{\text{sat}}I_{\text{phe}}/{}^{\text{sat}}I_{\text{PEP}}$.

RESULTS

Ligand binding isotherms - Isothermal fluorescence titrations (IFT)

In order to gain further insights to the allosteric regulation of RMPK, equilibrium titrations of RMPK by its ligands were performed to define ligand binding isotherms. All titrations were executed in the temperature range between 4 °C and 45 °C. At each temperature the titration curve was normalized by fluorescence intensity of the unliganded RMPK, I_0 . The normalization allowed eliminating a temperature dependence of the fluorescence intensity of the unliganded RMPK. Direct comparison of the shape of these binding curves was then possible.

PEP binding—Titration curves of RMPK with PEP at different temperatures are shown in Fig. 2. Visual inspection of the figure indicates that the fluorescence quantum yield of the R-state is lower than that of the T-state as evident by a gradual increase of I_{PEP}/I_0 at low temperatures and a sharp decrease above 30°C where the fraction of the unliganded T-state is known to significantly increase (1,4,30). Due to the preferential affinity of PEP for the R-state, the presence of PEP shifts the enzyme population toward the R-state resulting in a decrease of fluorescent intensity. Because the fraction of T-state of the unliganded RMPK increases with increasing temperature, a larger PEP-induced fluorescent decrease was observed at elevated temperatures. The small fluorescence increase recorded at low temperature, where RMPK is fully in the R-state, is an evidence for an additional binding-induced perturbation of the fluorescence quantum yield that is superimposed on the more significant fluorescence change caused by the $R \leftrightarrow T$ equilibrium shift. Because there are no indications of cooperativity in this region of small fluorescence increase, the change can be assigned to a direct perturbation of the Trp microenvironment by PEP binding at the level of the RMPK subunit.

Phe binding—Fluorescence changes induced by the binding of the inhibitor Phe at different temperatures are shown in Fig. 3. At all temperatures, increasing Phe concentration induces a

gradual increase in the I_{Phe}/I_0 ratio. The increase reaches a plateau at saturation. Fig. 3 shows that the plateau intensity, as well as the sigmoidicity of the curve, significantly decreases with increasing temperature. These data confirm that fluorescence quantum yield of the R-state is lower than that of the T-state. The preferential affinity of Phe for the T-state results in a shift of the RMPK population toward the T-state when Phe concentration increases. The increase is therefore accompanied by increasing fluorescent intensity. This is evident especially at low temperatures when RMPK is fully in the “low emission” R-state. A temperature elevation shifts the $R \leftrightarrow T$ equilibrium toward the T-state. As a consequence, smaller fraction of RMPK can be converted to the T-state by Phe binding and the magnitude of intensity increase becomes less pronounced. The sigmoidal shape of the IFT curves is in good agreement with the proposed concerted nature of the $R \leftrightarrow T$ transition. Upon binding of a single Phe molecule to the tetrameric RMPK causes concerted transition of all enzyme subunits from the active to the inactive state which has a significantly higher binding affinity for Phe. Binding of Phe to these high-affinity binding sites therefore becomes apparent after a single Phe binds to the unliganded RMPK in the R-state. As a consequence, a sigmoidal binding curve is observed. The observed fluorescent changes are qualitatively in good agreement with the two-state concerted model proposed for the allosteric regulation of RMPK (1–4,12).

Structural perturbation - Fluorescence temperature scans (FTS)

Effect of PEP—Fig. 4 shows detailed temperature dependence of the $^{sat}I_{PEP}/I_0$ ratio at a saturating PEP concentration (2mM). The profile qualitatively resembles the shape of the calorimetric data for PEP binding, shown in the previous paper (1).

Effect of ADP—Similarity between the ITC and the fluorescence data is observed also for the temperature scan in the presence of 10 mM ADP, as shown in Fig. 5. A decrease of the $^{sat}I_{ADP}/I_0$ ratio in Fig. 5 indicates activation of RMPK by ADP at temperatures between 30 and 40 °C. Such an observation is caused by shifting of the $R \leftrightarrow T$ equilibrium to the R-state which is characterized by low quantum yield. Subsequent fluorescent increase above 40 °C indicates that the ADP-induced activation is out weighed by a shift of the $R \leftrightarrow T$ equilibrium to the T-state. The observed increase is not a result of an irreversible thermal denaturation because the observed fluorescence changes were always reversible as indicated by a return to the initial signal with a reversal of the temperature. Indeed, the titration curves from the calorimetric and fluorescence experiments should resemble each other because these experiments are the same with the only difference being the method of detection.

Effect of Phe and ADP—The published ITC data (1) as well as the fluorescence experiments shown in this study prove that ADP exhibits temperature dependent differential affinity for the R and T-states; therefore its binding influences the $R \leftrightarrow T$ equilibrium. An antagonism between Phe and ADP was detected by the published ITC data (1). However, it is important to secure independent data to test the validity of the conclusions derived from the ITC data. Results of our fluorescence measurements can provide the necessary information. Since the fluorescence quantum yield of the R-state is lower than that of the T-state, shift of the $R \leftrightarrow T$ equilibrium can be monitored by fluorescence intensity. If the enzyme in the presence of ADP and saturating concentration of Phe was in the T-state at all temperatures, then the $^{sat}I_{ADP,Phe}/^{sat}I_{Phe}$ ratio should be essentially independent of temperature and ADP concentration. Figure 6 shows a temperature dependence of the $^{sat}I_{ADP,Phe}/^{sat}I_{Phe}$ ratio at a saturating Phe concentration of 12 mM and three different concentrations of ADP. At low ADP concentration (1 mM) the $^{sat}I_{ADP,Phe}/^{sat}I_{Phe}$ ratio is temperature independent, indicating that 1 mM ADP does not exert any significant effect on the redistribution of the R and T-states. At 2 mM ADP, however, the ratio starts out at a lower value at low temperature. At 10 mM ADP a significant decrease was observed. Data from Fig. 6 suggest that at low temperatures the ADP binding overcomes the inhibiting effect of Phe by shifting a fraction of RMPK to the R-state. This is a consequence

of both differential affinity of ADP and an altered differential affinity for Phe for the R- and T-state.

Parameterization of model—In the fluorescence model described in the Methods section it was assumed that each multiply liganded R-state (R_{ij}) and T-state (T_{ij}) has a unique fluorescence quantum yield and, as a consequence, the I/I_0 ratio can be characterized by a normalized fluorescent coefficients $\tilde{a}_{ij}$ and b_{ij} , respectively, see eq. (10) and eq. (11). Indices i and j denote the number of substrate and inhibitor molecules, respectively, bound to the RMPK tetramer. The FTS curve shown in Fig. 7 provides the data to indicate that these coefficients are temperature dependent. If the coefficients $\tilde{a}_{ij}$ and b_{ij} were temperature independent, the measured $^{sat}I_{Phe}/^{sat}I_{PEP}$ ratio should be constant as suggested by eq. (15). Instead, a decrease of this ratio with increasing temperature was observed. Temperature dependence of the $^{sat}I_{Phe}/^{sat}I_{PEP}$ ratio implies that parameters $\tilde{b}_{00}$, ξ_{PEP}^R , and ξ_{Phe}^T from eq. (15), or some of them, are temperature dependent. This conclusion is not surprising because proteins are dynamic structures where an extent of the internal mobility depends on temperature. Therefore, ligand-induced conformational changes of the RMPK structure perturbing the fluorescence response can be temperature dependent. Not having *a priori* information about the functional form of this dependence, it was incorporated into the model as a quadratic function of temperature:

$$\tilde{b}_{00} = {}^0\tilde{b}_{00} + {}^1\tilde{b}_{00} \cdot T + {}^2\tilde{b}_{00} \cdot T^2 \quad (16)$$

$$\xi_{\text{ligand}}^{\text{state}} = {}^0\xi_{\text{ligand}}^{\text{state}} + {}^1\xi_{\text{ligand}}^{\text{state}} \cdot T + {}^2\xi_{\text{ligand}}^{\text{state}} \cdot T^2 \quad (17)$$

To keep the model as simple as possible, the coefficients ξ_{PEP}^T and ξ_{Phe}^R ($i = 0, 1, 2$) associated with the 'weak binding' of PEP to the T-state and Phe to the R-state were set to zero at all temperatures since these binding constants are small.

Global fitting of calorimetric and fluorescent data sets

Quality of fit: Having the model properly parameterized we performed a global fit of all curves from Fig. 2, Fig. 3, Fig. 4 and Fig. 7. In order to “over” determine the model as much as possible (i.e. the same parameter was experimentally defined by multiple techniques under a variety of conditions), the full set of the ITC curves from the previous paper (1) was included in the analysis. Such combination allows for better verification of the model and for more accurate recovery of model parameters. The target parameters in the analysis were enthalpies and entropies of the characterized equilibrium reactions, linked protonations, and fluorescent coefficients. Results are summarized in Table 1. Where applicable, values from the calorimetry paper were used as an initial guess (1).

The best global fit is presented in Fig. 4, Fig. 6, Fig. 8, and Fig. 9 by solid lines¹. The reproducibility of the fluorescence measurements within weeks with the same batch of the protein was about 2%. Reproducibility of the experiments within months and different batches of RMPK was about 6 – 10 %. Examination of the quality of the fit reveals good agreement of the model with the measured data taking into account that 27 curves were simultaneously fitted. Some systematic deviations of the fit from the PEP titration data, as shown in Fig. 9, are of the order of the experimental reproducibility. The fit in Fig. 9 cannot simply be corrected by an

¹Fit of the ITC data resulting from this global fitting is shown in the previous paper 1. Herman, P., and Lee, J. C. Functional Energetic Landscape in the Allosteric Regulation of Muscle Pyruvate Kinase I. Calorimetric Study, *Manuscript 1*.

additional parameters, e.g. by incorporation of a nonzero heat capacity change for PEP binding, $\Delta C_{p, PEP}$. Addition of $\Delta C_{p, PEP}$ as a fitting parameter yielded rather small value of $-0.03 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{deg}^{-1}$ without noticeable improvement in the fit. The cause of such systematic deviations could be a more complex dependence of the fluorescent intensity on temperature and on the ligation state. A resolution of this issue will depend on additional data from another approach.

17 out of the 21 thermodynamic parameters obtained by global fitting of both the ITC and the fluorescence data are in a good agreement with the parameters obtained by global fitting of calorimetry data alone (1). Even for the four remaining thermodynamic parameters, namely, ΔS_{Phe}^R , ΔH_{Phe}^R , $\Delta \Delta S_{\text{ADP,Phe}}^T$ and $\Delta \Delta S_{\text{ADP,Phe}}^T$, the differences are within the uncertainties estimated for the ITC data alone.

Parameters derived from global fit: R→T transition: Table 1 shows that an unfavorable enthalpy change of about 50 kcal/mol of enzyme is associated with the temperature dependent R→T transition. The state transition is therefore driven by a large positive entropy change of about 154 cal/K/mol of enzyme. This observation is in full agreement with the fact that the inactive T-state assumes a looser, less ordered conformation deduced from data derived from analytical ultracentrifugation and sulfhydryl titrations(12), equilibrium gel titrations (13), and small angle neutron scattering(14). Steady-state kinetic data also indicate that the R→T transition could be entropy driven(4). The structural R→T transition was found to be accompanied by an increase in the isobaric heat capacity change $\Delta C_{p, R\rightarrow T} = 2.2 \text{ kcal/K/mol}$ of tetramer, Table 1. Very large increases of ΔC_p are generally associated with a reversible unfolding of proteins (31,32). These increases are attributed mainly to the exposure of hydrophobic groups to the solvent and to a consequent reordering of the neighboring water molecules (33,34). A significant contribution to the observed $\Delta C_{p, R\rightarrow T}$ increase can be attributed to the solvation of hydrophobic groups. It seems unlikely that ionization of RMPK groups would contribute to the observed $\Delta C_{p, R\rightarrow T}$, since less than one proton was found to be absorbed during the R→T transition and ΔC_p of protein groups ionization was reported to be about $\pm 50 \text{ cal/K/mol}$ (35). At 23 °C the resulting free energy change for the R→T transition can be estimated to be $\sim 3.4 \text{ kcal/(mol of tetramer)}$, decreasing to zero at 38 °C, which is close to a rabbit body temperature (36–38). Significance of this finding is discussed in the next paper (30).

Phe binding: Results from Table 1 indicate that Phe binding to either the T or the R-state of RMPK are entropically favored with an enthalpy change close to zero. This is a typical situation for an association governed mainly by a change in the solvation state resulting from a release of bound water from the protein or from the ligand. In this case it is most likely induced mainly by hydrophobic interactions that become stronger at higher temperatures (39). Due to the very small values for ΔH_{Phe}^R and ΔH_{Phe}^R , the Phe binding exhibits only very weak temperature dependence.

PEP binding: Binding of PEP to the R-state was found to be both entropy and enthalpy driven with ΔS_{PEP}^R and ΔH_{PEP}^R of 11.2 cal/mol/K and -2.1 kcal/mol , respectively. Although electrostatic interactions are expected to dominate, hydrogen bonds can also stabilize the RMPK/PEP complex. Since each hydrogen bond is expected to contribute about 2 kcal/mol to the enthalpy term (39) a speculation is that one hydrogen bond could be involved in the complex formation. Due to very weak binding of PEP to the T-state the parameters characterizing this interaction were not accessible from the data with reasonable accuracy. In an attempt to fit enthalpy and entropy terms for the binding of PEP to the T-state, no well-defined minimum of the global χ^2 for those parameters was found. The program converged with difficulty returning divergig

non-physiological K_{PEP}^T values. When the χ^2 profile was sampled by fixing the K_{PEP}^T to different values, the χ^2 decreased with increasing K_{PEP}^T finally becoming insensitive to any further increase of K_{PEP}^T . The quality of fit was essentially the same whether K_{PEP}^T was a fitting parameter or fixed to a value simulating no binding at all. Consistent with literature, this result indicates that the affinity of PEP for the T-state is much weaker than that to the R-state (4).

ADP binding: Table 1 shows that the binding of ADP to both the R and T state is enthalpy driven. The favorable enthalpy decrease is compromised by a relatively large entropy loss. The theoretical limit of about a decrease of 35 cal/mol/K in entropy is normally attributed to a full restriction in motional freedom of a ligand in the complex (39). Large negative entropy terms found for ADP binding suggest that the contacts between bound ADP and the enzyme significantly restrict the motion of the side chains in the RMPK/ADP complex. Due to the difference in ΔH_{ADP}^R and ΔH_{ADP}^T the ADP dissociation constants exhibit different temperature dependence (eq. 5). As a consequence, differential affinity of ADP for the R and T-state varies with temperature. From values given in Table 1 it can be calculated, that this differential activity is zero at temperature near 12 °C where the presence of ADP does not induce shifts in the R $\leftrightarrow$ T equilibrium. At higher temperatures ADP binds stronger to the R-state causing activation of the enzyme by shifting the R $\leftrightarrow$ T equilibrium toward the active R-state. At temperatures below 12 °C an inactivating effect of ADP is to be expected.

Coupling between ADP and Phe binding: The presence of ADP leads to decrease in the binding enthalpy and entropy for Phe, $\Delta\Delta H_{\text{ADP,Phe}}^T = -0.54 \text{ kcal}\cdot\text{mol}^{-1}$ and $\Delta\Delta S_{\text{ADP,Phe}}^T = -4.9 \text{ cal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$, respectively. The decrease indicates that the “ordering effect” induced by ADP is sensed by Phe. Interestingly, in the presence of ADP the small positive enthalpy term ΔH_{Phe}^T diminishes, $\Delta H_{\text{ADP,Phe}}^T = (\Delta H_{\text{Phe}}^T + \Delta\Delta H_{\text{ADP,Phe}}^T) = (0.65 - 0.54) \text{ kcal/mol} = 0.11 \text{ kcal/mol}$, rendering the binding of Phe to the T-state essentially temperature independent. At room temperature the interaction free energy of Phe and ADP can be calculated as $\Delta\Delta G_0^T = -540 + 300 \cdot 4.9 = +0.9 \text{ kcal/mol}$. This result implies that binding of these ligands in the presence of each other is unfavorable. The value is comparable with the antagonistic interaction energy between Phe and PEP of +1.2 kcal/mol that was previously found from steady-state kinetic data (12).

DISCUSSION

For the four linked reactions that characterize PK function, at least 21 thermodynamic parameters are required to adequately define the network of linked interactions involved in regulating the allosteric behavior of RMPK. The only chance to define these parameters with accuracy is to over-determine these parameters with multiple sets of data acquired by different approaches. The ITC data are most useful, however, in order to garner more data points to enhance the chance to accurately define these parameters we employed an additional technique which provide structural information that is sensitive to both ligand binding and conformational changes. A comparison of the results summarized in Table 1s in the previous paper (1) and this work shows clearly the power of multiple approaches and global fitting.

In order to validate and extend the model established in the previous paper (1), some of its assumptions were challenged. Similar to the calorimetry data, we attempted to fit the IFT data with $\Delta C_{p,R\rightarrow T}$ fixed to zero. Under these conditions the fitting program never converged. This indicates inconsistency of $\Delta C_{p,R\rightarrow T} = 0$ not only with the ITC data, but with the fluorescence data, too. After letting $\Delta C_{p,R\rightarrow T}$ be a parameter to be defined, the fitting converged to the values presented in Table 1.

We also verified the coupling between the interactions involving Phe and ADP reported in the previous paper (1). An analysis of the full data set without accounting for the ADP-Phe interaction (the $\Delta\Delta H_{ADP,Phe}^T$ and the $\Delta\Delta S_{ADP,Phe}^T$ terms were fixed to zero) led to fits with unacceptable statistics. This confirms the presence of an antagonistic coupling between ADP and Phe binding.

In addition to the thermodynamic parameters, 9 spectroscopic parameters were derived from the fluorescence data providing structural characterizations of the R and T-state. In order to further challenge the fitted model from the viewpoint of structural information, fluorescence parameters from Table 1 were used to simulate dependence of the $^{sat}I_{ADP}/I_0$ ratio on temperature. The result calculated according to eq. (10) is shown in Fig. 5 by the solid line. At temperatures between 30 and 45°C the simulation predicts a decrease of the $^{sat}I_{ADP}/I_0$ ratio that results from the activation of RMPK which is present in a higher population of the less fluorescent R-state due to the higher binding affinity of ADP to the R-state.. Fig. 5 shows that the simulation is in a good qualitative and quantitative agreement with the measured data. Because the experimental data from Fig. 5 were not used for the fitting, the agreement between the prediction and the experimental data is a fully independent test of the validity of the model. It is most interesting to note that the ratio of fluorescence intensity reaches a minimum at ~40°C indicating that the optimal temperature for 10 mM of ADP to switch RMPK to the active R-state is at 40°C. However, at higher temperature the trend is reversed. This indicates that the state distribution of RMPK is reversed in favor of the T-state. This reversal of behavior is the direct consequence of the favoring of the $R \leftrightarrow T$ equilibrium towards the T-state high at these higher temperatures.

The FTS data in Fig. 6 are to show the shift in the state of RMPK by temperature in the presence of various combinations of ADP and Phe concentrations. At 12 mM of Phe, RMPK is in the T-state which is characterized by high quantum yield. A decrease in the ratio of fluorescence intensity indicates a decrease of the fraction of T-state and an increase in the fraction of R-state which exhibits low quantum yield. The FTS data shown in Fig. 6A indicate that at low ADP concentrations of 1 mM ADP and 12 mM Phe there was no state change in RMPK induced by temperature. This is due to the fact that there was not enough ADP to bind to RMPK to affect the distribution of states in RMPK. At 2 mM ADP and 12 mM Phe, there was slight decrease in the fluorescence intensity ratio at low temperature implying a shift towards the R-state under those conditions. At 10 mM ADP and 12 mM Phe, there was a significant drop in the ratio at low temperature. The ratio approaches 1.0 at higher temperature. This behavior is a reflection of the effect of temperature on the $R \leftrightarrow T$ equilibrium and the reversal of the ratio of affinity of ADP for the R and T-state (See 'ADP binding in Results'). To test the validity of the derived parameters in Table 1, temperature dependence of the $^{sat}I_{ADP,Phe}/^{sat}I_{Phe}$ ratio in the presence of 12 mM Phe and 10 mM ADP was simulated as depicted in Fig. 6B. The curve was calculated using eq.(10) twice and making ratio $^{sat}I_{ADP,Phe}/^{sat}I_{Phe} = (^{sat}I_{ADP,Phe}/I_0)/(^{sat}I_{Phe}/I_0)$. It is obvious that the predicted dependence is in a good qualitative agreement with the experimental data shown in Fig. 6A. When the simulation was performed by neglecting the ADP-Phe coupling, the simulated $^{sat}I_{ADP,Phe}/^{sat}I_{Phe}$ ratio was unity at all temperatures. This did not agree with the measured data. Since the FTS data from Fig. 6A were not included in the global fitting, the observed agreement between the predicted and the measured effects strongly supports both the validity of the fitted model and the conclusions about the role of ADP in the allosteric regulation of RMPK.

Upon closer examination, there is an apparent discrepancy between data shown in Fig. 5 and Fig. 6. While in Fig. 5 the activating effect of ADP is most pronounced at high temperatures and almost no effect is seen below 30 °C, in Fig. 6 the opposite was observed. In the presence of Phe, activation of RMPK was observed at low temperatures and the effect decreased with increasing temperature. The clue is in the high temperature dependence of the equilibrium

constant L_0 describing the $R \leftrightarrow T$ equilibrium. Using the parameters in Table 1 it can be shown that more than 96% of RMPK are in the active state at 30 °C or lower temperatures and the activating effect of ADP is therefore insignificant, as shown in Fig.5. At 45 °C the $R \rightarrow T$ transition is almost complete and the activation by ADP does not dominate over this $R \rightarrow T$ transition. The combined effect of temperature and Phe inhibition leads to no observable effect of ADP in the presence of Phe above 30 °C, Fig. 6.

In conclusion, complementation of the fluorescence experiments with the ITC data(1) allowed for characterization of multiple linked equilibria associated with an allosteric regulation of the RMPK. Global data analysis played an essential role in this process since we could quantify parameters that would not be easily accessible by other means. Importantly, we confirmed an increase of the isobaric heat capacity associated with the entropy driven $R \rightarrow T$ transition and an intrigue role of ADP in the enzyme activation process. A graphical representation of current results, their physiological relevance, and simulated predictions of the RMPK allosteric regulation is presented in the next paper (30).

Remarks on global fitting

Non-linear least square data analysis is often used for validation of theoretical model that describes data under investigation since it looks for an objective comparison between the experimental data and the proposed model (19). As the model complicates and number of model parameters increases the fitting problem starts to be ill-defined because data do not carry enough information to accurately recover all parameters. In other words, the minimum of the χ^2 surface starts to be too shallow and correlation between parameters becomes too high to find correct parameter values with confidence. Very powerful approach that helps to sharpen χ^2 minimum and to remove correlation between parameters is a simultaneous analysis of multiple data sets acquired under different experimental conditions and/or with different techniques - the global analysis (17,21). The global analysis is an extension of a conventional least square fitting for simultaneous analysis of multiple data sets under a single model that encompasses them all. Important feature of this approach is that model parameters may be common for different curves. Such parameter linkage over determines the model and sharpens the χ^2 surface (17). The global analysis has been previously used for a large variety of different experimental data and experimental techniques (1,2,18,22–26,40,41). In all cases it provided performance far beyond resolution of the conventional analysis.

Our model needs 30 parameters in order to describe allosteric regulation of RMPK in the presence of the three examined ligands, Table 1. This number is definitely too high to recover them all from a single experiment. Fortunately, under carefully chosen experimental conditions, e.g. in the presence of single ligands, the number of parameters analytically describing the individual curves significantly decreases. It is not unusual in literature to analyze single experimental curve containing a dozen of points by a 3 – 5 parameter model. *In this study 27 independent curves with more than 1600 experimental points were globally analyzed.* From this point of view the current statistics of about 1.1 parameter per curve and 55 data points per parameter is rather favorable and allows for good model validation. Moreover, many parameters are common for multiple data sets. For instance, parameters $S_{0,R \rightarrow T}$, $H_{0,R \rightarrow T}$, and $\Delta C_{p,R \rightarrow T}$ characterizing the $R \leftrightarrow T$ equilibrium are common for 26 of 27 curves and due to high over determination they were recovered with good accuracy.

During the data analysis we became aware that the multidimensional χ^2 surface contains local minima. To avoid being trapped in such minimum, the fitting was initiated with the limited data set (and limited number of parameters) from isothermal calorimetry. The known parameters were fixed and fluorescence curves were added individually or in groups with subsequent fitting. After optimization, all parameters were released and the system was optimized again. This procedure was repeated for several different sequences with expansion

of global data set. This procedure always resulted with the same final set of the parameter values. The global fitting with numerous sets of the initial parameter guesses were performed to make sure that the fitting converges to the same global minimum.

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Fig 1. Structure of RMPK. Color and domain assignments are: blue, B (residues 116 – 218); red, A (residues 12 – 115; 219 – 387); yellow, C (residues 388 – 530); green sphere, active site; cyan spheres, tryptophan residues 157 in domain B, residues 481 and 514 in domain C). (PDB: 1F3W)

Fig. 2.
Normalized fluorescence intensity of RM PK as a function of temperature and PEP concentration.

Fig. 3. Normalized fluorescence intensity of the RMPK as a function of temperature and Phe concentration.

Fig. 4. Fluorescence intensity of RMPK saturated by 2mM PEP relative to the fluorescence intensity of the unliganded enzyme. The solid line represents the best overall global fit.

Fig. 5. Temperature dependence of the RMPK fluorescence intensity in the presence of 10 mM ADP normalized by the fluorescence intensity of the unliganded enzyme. Solid line is a dependence predicted by the model.

Fig. 6.

Fluorescence temperature scans: (A) Fluorescence intensity of RMPK saturated by 12 mM Phe in the presence of 1 mM ADP (green), 2 mM ADP (red), and 10mM ADP (blue). The intensities are normalized by the fluorescence intensity of RMPK saturated by 12 mM Phe. (B) Predicted temperature dependence of $^{sat}I_{ADP,Phe}/^{sat}I_{Phe}$ calculated from model parameters obtained by global fitting.

Fig. 7. Fluorescence intensity of RMPK in the presence of 2 mM PEP relative to the fluorescence intensity of RMPK saturated by 12 mM Phe. The solid line represents the best overall global fit.

Fig. 8. Fluorescence titration curves of RMPK titrated by Phe at 15°C (○), 20°C (●), 25°C (▽), 30°C (▼), 32°C (□), 35°C (■), 37.5°C (△), and 40°C (▲). Solid lines represent the best overall global fit.

Fig. 9.
Fluorescence titration curves of RMPK titrated by PEP at 10°C (○), 15°C (●), 20°C (▽), 31°C (▼), 32.5°C (□), 34.5°C (■), 37.5°C (△), 39°C (▲), and 40°C (◇).

Table I

Parameters obtained from global fitting of the IFT, FTS and ITC* data sets.

Reaction	Parameter	Value	Unit ^a
R $\leftrightarrow$ T	$\Delta S_{0,R\rightarrow T}$	154 (3) ^{c, e}	cal·mol ⁻¹ ·K ⁻¹ ^b
	$\Delta H_{0,R\rightarrow T}$	49 (4) ^c	kcal·mol ⁻¹ ^b
	$\Delta C_{p,R\rightarrow T}$	2.2 (0.2) ^c	kcal·mol ⁻¹ ·K ⁻¹ ^b
	$\Delta n_{R\rightarrow T}$	0.7 (0.1) ^d	mol ⁻¹ ^b
Phe binding	ΔS_{Phe}^R	2.7 (0.9)	cal·mol ⁻¹ ·K ⁻¹
	ΔH_{Phe}^R	-1.4 (0.4)	kcal·mol ⁻¹
	ΔS_{Phe}^T	18.2 (0.2)	cal·mol ⁻¹ ·K ⁻¹
	ΔH_{Phe}^T	0.65 (0.20)	kcal·mol ⁻¹
	Δn_{Phe}^T	-1.0 (0.1)	mol ⁻¹
PEP binding	$\Delta S_{0,PEP}^R$	11.2 (0.2)	cal·mol ⁻¹ ·K ⁻¹
	$\Delta H_{0,PEP}^R$	-2.1 (0.3)	kcal·mol ⁻¹
	Δn_{PEP}^R	0.18 (0.03)	mol ⁻¹
ADP binding	ΔS_{ADP}^R	-20 (2)	cal·mol ⁻¹ ·K ⁻¹
	ΔH_{ADP}^R	-10.5 (1.0)	kcal·mol ⁻¹
	Δn_{ADP}^R	0.75 (0.02)	mol ⁻¹
	ΔS_{ADP}^T	-28 (2)	cal·mol ⁻¹ ·K ⁻¹
	ΔH_{ADP}^T	-12.8 (1.0)	kcal·mol ⁻¹
	Δn_{ADP}^T	0.98 (0.02)	mol ⁻¹
ADP-Phe coupling	$\Delta\Delta S_{ADP,Phe}^T$	-4.9 (0.5)	cal·mol ⁻¹ ·K ⁻¹
	$\Delta\Delta H_{ADP,Phe}^T$	-0.54 (0.30)	kcal·mol ⁻¹
	$\Delta n_{ADP,Phe}^R$	-0.9 (0.5)	mol ⁻¹
Fluorescence parameters	${}^0b_{00}$	227 (3)	
	${}^1b_{00}$	-1.48 (0.01)	
	${}^2b_{00}$	2.43 (2.00)×10 ⁻³	
	${}^0\zeta_{PEP}^R$	1.80 (0.02)	
	${}^1\zeta_{PEP}^R$	-1.22 (0.01) × 10 ⁻²	
	${}^2\zeta_{PEP}^R$	2.06 (3.00) × 10 ⁻⁵	
	${}^0\zeta_{Phe}^T$	-57.3 (0.3)	
	${}^1\zeta_{Phe}^T$	3.78 (0.01) × 10 ⁻¹	
	${}^1\zeta_{Phe}^T$	-6.22 (14.00) × 10 ⁻⁴	

* Isothermal titration calorimetry data published in the previous paper were used together with the fluorescence data for global fitting (1)

^a Per mol of a ligand

^b Per mol of tetramer

^c For $T_0=20^{\circ}\text{C}$. T_0 was a fixed parameter.

^d Amount of protons absorbed (positive value) or released (negative value). Buffer ionization heats are 11.3 kcal/mol and 6.8 kcal/mol for TKM and BTKM, respectively (42)

^e Standard deviations are given in parenthesis.