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Kinetics of Creatine Kinase in Heart: A ^{31}P NMR Saturation- and Inversion-Transfer Study†

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Received October 25, 1984

ABSTRACT: The kinetics of the phosphate exchange by creatine kinase (CK) was studied in solution and in the Langendorff-perfused rat heart at 37 °C. ^{31}P inversion-transfer (IT) and saturation-transfer (ST) methods were applied. The kinetic parameters obtained by the two magnetization transfer methods were the same, whether in solution or in the perfused heart. Inversion transfer is the more efficient method, yielding the kinetic constants for the exchange and the relaxation rates of the transferred phosphate in both substrates, in one experiment. In solution the forward (k_F) and reverse (k_R) pseudo-first-order rate constants for the CK reaction ($k_F = k_1$[MgADP][H^+]; $k_R = k_{-1}$[creatine]) as well as the concentrations of phosphocreatine (PCr), MgATP, and creatine (Cr) remained constant between pH 6.9 and pH 7.8. Equilibrium at this pH region is therefore maintained by compensating changes in the concentration of MgADP. The forward and reverse fluxes in the perfused heart were equal with an average flux ratio ($flux_F/flux_R$) of 0.975 ± 0.065 obtained by both methods. Average values of k_F and k_R were 0.725 ± 0.077 and 1.12 ± 0.14 s^{-1}, respectively. These results clearly indicate that the CK reaction in the Langendorff-perfused heart is in equilibrium and its rate is not limited by the diffusion of substrates between different locations of the enzyme. There is therefore no indication of compartmentation of substrates of the CK reaction.

The function of creatine kinase (CK) in myocardial cells is to catalyze the transfer of a high-energy phosphate between ATP and phosphocreatine (PCr). The reaction has an apparent equilibrium constant $K_{app} = K_{eq}[H^+] = [MgATP][Cr]/([PCr][MgADP])$ of 166 at pH 7, 38 °C, ionic strength 0.25, and 1 mM Mg^{2+} (Lawson & Veech, 1979). Its rates in myocardial cells appear to be much higher than those of the myocardial ATPase or ATP synthetase reactions (Matthews et al., 1982). Therefore, when ADP ≪ ATP, as in heart, CK and the PCr pool can provide a high-energy phosphate buffer that serves to maintain fairly constant levels of ATP even during heavy periods of ATPase activity. Of the total concentration of CK, approximately 20% is known to exist as the MM isozyme bound to the myofibrils and 40% as another isozyme bound to the outside of the inner mitochondrial membrane. The remainder is found free in the cytoplasm or bound to other organelles [for a review, see Saks et al (1978)].

†Supported by NIH Grant AM 27121 and NSF Grant PCM 80 21715.
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0006-2960/85/0424-5510$01.50/0

ATP synthesized in the mitochondria is used preferentially for creatine phosphorylation (Moreadith & Jacobus, 1982), and this PCr is subsequently used to produce the ATP that is cleaved by the myofibrillar ATPases (Bessman et al., 1980). Consequently, the CK–PCr system can be considered to provide an energy-transport or "shuttle" system between the sites of ATP production and hydrolysis [for a review, see Bessman & Geiger (1981)]. As recently pointed out by Meyer et al. (1984), this role would be a natural consequence of the reaction being near equilibrium everywhere in the cell.

The fluxes through the reaction catalyzed by CK in a Langendorff-perfused heart have been previously measured by employing ^{31}P NMR saturation-transfer techniques (Brown et al., 1978; Nunnally & Hollis, 1979; Matthews et al., 1982). In the first study, the range of the reported reverse flux through CK (ATP → PCr) was too large to determine its relationship to the forward flux (PCr → ATP). In the latter two studies, the forward flux was found to exceed the reverse flux although the ATP and PCr levels remained constant with time (Nunnally & Hollis, 1979; Matthews et al., 1982). Two explanations were suggested for this apparent discrepancy: (1) the presence of other reactions that interconvert ATP and ADP at rates comparable to, or faster than, CK (Matthews et al., 1982) and (2) two or more nonequivalent cellular pools of ATP where the reaction is at equilibrium but may proceed at different rates (Matthews et al., 1982; Nunnally & Hollis, 1978). The latter supports the "shuttle" model where newly synthesized ATP in the mitochondria is used for the manufacture of PCr that transports the high-energy phosphate by diffusion to other localized pools of CK where ATP is regenerated and hydrolyzed. The circuit is completed by diffusion of creatine back to the mitochondria (Saks et al., 1978).

A close look at the saturation-transfer data mentioned above led us to conclude that although the forward and reverse fluxes appear to be statistically different, the inherent systematic errors associated with the time-dependent saturation transfer (Nunnally & Hollis, 1978) and steady-state saturation transfer measurements (Brown et al., 1978; Matthews et al., 1982) and errors associated with determining the individual kinetic constants (the rate constants and T_1 values) on separate hearts are too great to allow the conclusions that had been made. Therefore, we have decided to study the CK kinetics in perfused rat heart and in solution by employing the inversion-transfer method that was previously used to measure kinetics of chemical reaction (Alger & Prestegard, 1977; Campbell, 1978) and in vivo enzyme kinetics (Brown et al., 1980) and to compare the results with those obtained by steady-state saturation-transfer experiments on similar hearts. Inversion transfer has the advantage of allowing the determination of the forward and reverse fluxes of the CK reaction as well as the longitudinal relaxation rate of the transferred phosphate of ATP (γ) and PCr in the same heart.

Theory

Kinetics. The chemical reaction catalyzed by creatine kinase (CK) is

$$PCr^{2-} + MgADP^{-} + H^{+} \rightleftharpoons Cr + MgATP^{2-} \quad (1)$$

The forward and reverse pseudo-first-order rate constants for the exchange of the phosphate between MgATP and PCr are

$$k_F = k_1[MgADP^{-}][H^{+}] \quad (2)$$

$$k_R = k_{-1}[Cr] \quad (3)$$

The corresponding fluxes are therefore

$$\text{flux}_F = k_F[PCr^{2-}] \quad (4)$$

$$\text{flux}_R = k_R[MgATP^{2-}] \quad (5)$$

Inversion Transfer. In the inversion-transfer experiment of the CK reaction the time course of the recovery of the ^{31}P signals of PCr and γ-ATP following selective inversion of either phosphate peak is monitored. Inverted magnetization is transferred to the undisturbed resonance through chemical exchange. The time-dependent behavior of both signals, inverted [$M^i(t)$] and noninverted [$M^a(t)$], is described by the complete solutions to the bloch equations modified to include the exchange (Alger & Prestegard, 1977):

$$M^i(t) = M^i(\infty) + c_1 \exp(\lambda_+ t) + c_2 \exp(\lambda_- t) \quad (6)$$

$$M^a(t) = M^a(\infty) + c_3 \exp(\lambda_+ t) + c_4 \exp(\lambda_- t) \quad (7)$$

where

$$\lambda_\pm = (1/2)[-(1/\tau_1{}^i + 1/\tau_1{}^a) \pm [(1/\tau_1{}^i - 1/\tau_1{}^a)^2 + 4k^{i\rightarrow a}k^{a\rightarrow i}]^{1/2}] \quad (8a)$$

$$c_2 = [(1/k^{a\rightarrow i})[M^i(0) - M^i(\infty)] \times (\lambda_+ + 1/\tau_1{}^i) + [M^a(\infty) - M^a(0)]]/[(\lambda_+ - \lambda_-)(1/k^{a\rightarrow i})] \quad (8b)$$

$$c_1 = M^i(0) - M^i(\infty) - c_2 \quad (8c)$$

$$c_3 = c_1(\lambda_+ + 1/\tau_1{}^i)/k^{a\rightarrow i} \quad (8d)$$

$$c_4 = c_2(\lambda_- + 1/\tau_1{}^i)/k^{a\rightarrow i} \quad (8e)$$

$1/\tau_1{}^i$ and $1/\tau_1{}^a$ are the apparent longitudinal relaxation rates of the inverted and noninverted nuclei, respectively, and include contributions from all relaxation processes.

Under equilibrium conditions for the CK reaction, the apparent relaxation rates of the exchanging phosphate in both substrate γ-ATP and PCr are defined by

$$1/\tau_1(ATP) = 1/T_1(ATP) + k_R \quad (9)$$

$$1/\tau_1(PCr) = 1/T_1(PCr) + k_F \quad (10)$$

The time-dependent behavior of the γ-ATP and the PCr signals during an inversion-transfer experiment are therefore dependent on the four kinetic constants, $1/\tau_1(ATP)$, $1/\tau_1(PCr)$, k_F, and k_R.

By performing the two time course experiments, one in which γ-ATP is inverted, and another with PCr inverted, the four unknown parameters are determined from the four independent solutions to the Bloch equations. However, analysis of the results of one time course experiment can yield all the four unknowns.

Saturation Transfer. In a saturation-transfer experiment of the CK reaction, one signal is saturated (γ-ATP or PCr) and the other is observed. The magnitude of the nonsaturated signal in a time-dependent saturation-transfer experiment (TDST) depends on the saturation time according to

$$M^a(t) = M^a(0)(1 - k^{a\rightarrow s}\tau_1{}^a[1 - \exp(-t/\tau_1{}^a)]) \quad (11)$$

where s and a indicate saturated and nonsaturated signals. As time approaches infinity ($5\tau_1$ s), a new equilibrium magnetization $M^a(\infty)$ is reached and the difference, $M^a(0) - M^a(\infty) = \Delta M^a$, in the steady-state saturation-transfer experiment (SSST) is given by

$$\Delta M^a = M^a(0)k^{a\rightarrow s}\tau_1{}^a \quad (12)$$

To characterize an exchange reaction by the rate constants, two saturation-transfer experiments are required as well as an independent measurement of the two relaxation rates. The pseudo-first-order rate constant $k^{a\rightarrow s}$ depends on the reciprocal value of the total magnetization $M^a(0)$ observed without

Table I: Kinetics of CK in Solution (37 °C) by Inversion Transfer[a]

pH	[PCr]/[MgATP]	$1/\tau_1$(PCr) (s^{-1})	$1/\tau_1$(ATP) (s^{-1})	K_F (s^{-1})	K_R (s^{-1})	% σ[b]	$flux_F/flux_R$
7.84	1.0	0.47	1.36	0.22	0.23	2.9	1.02
7.22	0.9	0.61	1.54	0.33	0.31	5.1	0.95

[a] The solution contained initially 50 mM Hepes, 68.4 mM KCl, 19.3 mM $Mg(C_2H_3O_2)_2$, 28.4 mM ADP, 57.7 mM PCr, and 1000 units of CK/mL. MgATP equals 85% of the total ATP and was determined by the method outlined under Materials and Methods. [b] % σ is defined under Data Analysis.

saturation. The flux through the enzyme depends on the total substrate concentration, which is proportional to $M^a(0)$. The flux is therefore proportional only to the change in magnetization ΔM^a, and the ratio of the fluxes is independent of the absolute values of the corresponding magnetizations.

Materials and Methods

Enzyme Solutions. Solutions for the in vitro studies initially contained 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 70 mM KCl, 30 mM ADP, either 60 or 70 mM PCr, and 32 mM or 19 mM magnesium acetate in 33% ^2H_2O by volume. All chemicals were of analytical grade. Creatine phosphokinase (EC 2.7.3.2.) from rabbit muscle, 100–150 units/mg of protein, was obtained from Sigma Chemical Co. and was used without further purification. The enzyme was added to the solutions at a concentration of 1000 units/mL, and the reaction mixture was allowed to equilibrate at 37 °C. The pH was measured with a Corning 125 pH meter to an accuracy of ±0.01 pH unit and adjusted between pH 6.88 and pH 7.94 with 1 N NaOH.

Animals. Male Sprague-Dawley rats (375–430 g) were anesthetized with 6.5 mg/100 g sodium pentobarbitol (Anthony Products, Arcadia, CA) and injected intravenously with 200 USP units of sodium heparin approximately 1 min before excision of the heart.

Heart Perfusion. The hearts were cannulated through the aorta by the Langendorff method and perfused at 37 °C with a bicarbonate Krebs–Henseleit buffer containing 118 mM NaCl, 4.7 mM KCl, 3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose, and 0.5 mM ethylenediaminetetraacetic acid (EDTA). The buffer was continuously bubbled with a 95% O_2 + 5% CO_2 gas mixture, and the pH was constant at 7.4 ± 0.002 (Garlick et al., 1979). The perfusion flow was sustained by a hydrostatic pressure of 100 cm of water. The hearts were placed in 20-mm NMR tubes. Aortic pressure and heart rate were recorded with a Gould–Statham physiological transducer in the delivery line. The stability of the perfused heart was monitored by repeatedly measuring the levels of PCr and ATP in fully relaxed ^{31}P NMR spectra. All experiments were completed within 4 h of cannulation. The PCr and ATP levels changed less than 20% during experiments.

NMR. ^{31}P spectra were recorded at 145 MHz with a Bruker WH-360 NMR spectrometer. Selective inversion was achieved by a DANTE pulse sequence (Morris & Freeman, 1978) consisting of 16–28 consecutive short pulses with a composite length corresponding to a 180° tilt angle at the frequency of the signal to be inverted. An interval of 0.240 ms placed the first harmonic well outside the high-energy phosphate region. Each inversion-transfer time course included 16–25 points with evolution periods between 0.002 and $3\tau_1$ s. These two time points were taken as controls at the beginning and end of each experiment. The ratio of the integrated intensities of the PCr and ATP signals were measured from fully relaxed control spectra taken before and after the experiments using both computer integration and peak weight. Steady-state saturation of either PCr or γ-ATP was achieved by a selective pulse (0.3 W) generated by a separate channel. Saturation was gated off during acquisition. Control spectra were obtained in an identical manner with the saturation frequency equidistant from, and on the other side of, the unsaturated resonance.

Data Analysis. The data obtained in each inversion-transfer (IT) time course were analyzed according to eq 6 and 7. The four kinetic constants, $1/\tau_1^i$, $1/\tau_1^a$, $k^{i\to a}$, and $k^{a\to i}$, were the adjustable parameters. Initial values of $M^i(0)$ and $M^a(0)$ were obtained experimentally by a short delay of 2 ms between the selective 180° pulse and the 90° pulse. Although an error in $M^a(0)$ could introduce a systematic deviation in the recovery of $M^a(t)$ (see upper right curve of Figure 4), we have found that in most cases the best fit was obtained with the experimental value of $M^a(0)$.

In each time course the intensity of the inverted resonance depends primarily on its own apparent relaxation rate ($1/\tau_1^i$). This relaxation rate is easily and accurately found from the initial slope of the inverted curve (Campbell et al., 1978) and is the most independent parameter of the four kinetic constants. In addition, it is important to note that due to the selective inversion the $1/\tau_1^i$ is accurately determined from the initial slope even when the inverted signal participates in several exchange processes. This usually occurs in vivo for γ-ATP that exchanges with both PCr and P_i. We have therefore used the values for $1/\tau_1^i$ of γ-ATP and PCr obtained in the IT experiments to analyze the saturation-transfer data.

The intensity of the noninverted resonance depends primarily on the pseudo-first-order rate constant of exchange into it ($k^{i\to a}$) and on its own apparent relaxation rate $1/\tau_1^a$. The reverse rate constant ($K^{a\to i}$) affects the decay of the inverted curve at times larger than τ_1^i. Each time course provides for the most accurate determination of the $1/\tau_1^i$ and $k^{i\to a}$ values.

The kinetic parameters were first obtained by computer simulation and visual fit of the inversion-transfer data to the general solutions of the Bloch equations modified to include chemical exchange (eq 6 and 7). These approximate kinetic values were used as initial estimates for a computer nonlinear best fit program that employs the modified Marquardt method of least squares, which yielded the final reported kinetic constants. The values are given with the average deviation of each data point from the calculated curve expressed as a percentage of the total PCr peak (σ).

The fraction of magnesium-bound ATP was determined from the difference in chemical shifts of α- and β-ATP ($\delta_{\alpha\beta}$) by using the constants $\delta_{\alpha\beta}$(ATP) = 10.8 ppm and $\delta_{\alpha\beta}$(MgATP) = 8.39 ppm according to the method of Gupta et al. (1978).

Results

Inversion-transfer and steady-state saturation-transfer experiments were performed in vitro on solutions of phosphocreatine kinase and its substrates. Representative spectra of the inversion-transfer time courses are shown in Figure 1. The PCr and γ-ATP inversion-transfer peak-height values were fitted to the modified Bloch equations as described above. Simulated curves calculated from the resultant kinetic constants plotted over the experimental data points are shown in Figure 2. The resulting kinetic constants are summarized in Tables I and II. The average deviation of each data point

Table II: Kinetics of CK in Solution (37 °C) by Saturation Transfer[a]

pH	[PCr]/[MgATP]	$\Delta M/M$(PCr)	$\Delta M/M$(ATP)	K_F (s^{-1})	K_R (s^{-1})	$flux_F/flux_R$
7.84	1.0	0.52	0.18	0.25	0.24	1.06
7.22	0.9	0.57	0.20	0.35	0.30	1.04

[a] The solution is the same as for Table I.

Table III: Kinetics of CK in Solution (37 °C) at Various pHs by Inversion-Transfer Experiments[a]

		s^{-1}					
pH	[PCr]/[MgATP]	$1/\tau_1$(PCr)	$1/\tau_1$(ATP)	K_F	K_R	% σ^b	$flux_F/flux_R$
7.94	1.35	0.42	1.35	0.17	0.25	2.0	0.93
7.54	1.35	0.46	1.34	0.18	0.24	2.0	0.97
7.23	1.35	0.47	1.41	0.21	0.27	2.1	1.05
6.88	1.40	0.47	1.31	0.16	0.28	1.1	0.80

[a] The solution contained initially 50.0 mM Hepes, 70.0 mM KCl, 31.9 mM $Mg(C_2H_3O_2)_2$, 30.0 mM ADP, 69.9 mM PCr, and 1000 units of CK/mL. [b] % σ is defined under Data Analysis.

Table IV: Kinetics of CK in Perfused Heart (37 °C) by Inversion-Transfer Experiments

		ATP inverted						PCR inverted					
heart no.	[PCr]/[MgATP]	$1/\tau_1$(PCr) (s^{-1})	$1/\tau_1$(ATP) (s^{-1})	k_F (s^{-1})	k_R (s^{-1})	% σ^a	$flux_F/flux_R$	$1/\tau_1$(PCr) (s^{-1})	$1/\tau_1$(ATP) (s^{-1})	k_F (s^{-1})	k_R (s^{-1})	% σ^a	$flux_F/flux_R$
1	1.4	0.81	2.56	0.74	1.02	5.1	1.02	0.95	2.51	0.75	1.02	6.5	1.03
2	1.5	1.08	2.57	0.69	1.13	7.3	0.92	1.07	3.63	0.91	1.44	5.6	0.95
3	1.6	1.10	2.50	0.65	1.25	5.5	0.83	1.10	2.30	0.95	1.60	4.6	0.95
4	1.4	0.90	2.60	0.74	1.00	11.8	1.04	0.90	2.60	0.74	1.00	15.7	1.04
5	1.7	1.30	2.30	0.89	1.50	3.8	1.01						
6	1.4	1.43	2.41	0.75	1.10	7.3	0.95						
7	1.3							0.97	2.81	0.74	1.01	4.5	0.95
8	1.4							1.18	2.80	0.84	1.20	13.9	0.98

[a] % σ is defined under Data Analysis.

from the calculated curve expressed as a percentage of total PCr peak height (σ) is between 1% and 5%. Steady-state saturation-transfer data were analyzed with eq 12 by using the apparent relaxation rates ($1/\tau_1$) found from the inversion-transfer data (Table I).

The two methods yielded similar forward and reverse pseudo-first-order rate constants. The forward and reverse fluxes were calculated from the determined rate constants and the ratio of concentrations of PCr and ATP: $flux_F/flux_R = (k_F/k_R)([PCr]/[MgATP])$.

The effect of varying the pH on CK kinetic constants was studied by employing the inversion-transfer method. Four experiments were performed at pH values ranging between 6.88 and 7.94. The pseudo-first-order rate constants do not vary significantly over the range of pH values studied (Table III).

Inversion-transfer (IT) and steady-state saturation-transfer (SSST) experiments were performed on Langendorff-perfused rat hearts as described above. Representative spectra of the inversion-transfer time course are shown in Figure 3. The dip in the noninverted peak is readily apparent. The peak heights of the PCr and ATP resonances are plotted in Figure 4 along with the computer-simulated curves obtained as described above. The average error associated with each point (σ) in the calculated curves was between 3.5% and 16% of total PCr peak height. Variation among the perfused hearts made it advantageous to present the experimental results individually. These kinetic constants derived from IT and SSST are shown in Tables IV and V, respectively. $flux_F/flux_R$ was calculated as described above. Steady-state saturation-transfer data (Table V) were analyzed by using the average apparent τ_1 values obtained from the inversion-transfer experiments (Table IV).

Pseudo-first-order rate constants and apparent relaxation rates varied from heart to heart, but the forward and reverse fluxes in each organ were found to be equal whether measured by inversion transfer or measured by saturation transfer.

Table V: Kinetics of CK in Perfused Heart (37 °C) by Saturation-Transfer Experiments

[PCr]/[MgATP]	$\Delta M/M$ (PCr)	$\Delta M/M$ (ATP)	K_F (s^{-1})[a]	K_R (s^{-1})[b]	$flux_F/flux_R$
1.5	0.64	0.43	0.68	1.14	0.89
1.5	0.65	0.37	0.70	0.99	1.06
1.4	0.59	0.37	0.63	0.98	0.90

[a] K_F and K_R were calculated by using $1/\tau_1$(PCr) = 1.07 s^{-1} and $1/\tau_1$(ATP) = 2.64 s^{-1}, respectively, obtained from averaging the inversion-transfer τ_1s.

Discussion

The solution NMR studies of the CK reaction under equilibrium conditions indicate that within experimental error the apparent first-order rate constants k_F and k_R do not change over the physiological pH range studied (6.9–7.9). Even though H^+ increases by 1 order of magnitude, no change is detected in the ratio of PCr to ATP. Since k_R and [Cr] do not change, it can be seen from eq 3 that k_{-1} and therefore also k_1 the true rate constants are independent of pH within the range studied. The kinetic mechanism of CK at pH 8 is a rapid equilibrium, random in both directions, with the actual chemical reaction of phosphate transfer as the rate-limiting step (Morrison & James, 1965; Morrison & Cleland, 1966; Engelborghs et al., 1975). At pH 7 phosphorylation of creatine proceeds in a rapid equilibrium ordered mechanism with MgATP adding to the enzyme prior to creatine (Schimerlik & Cleland, 1973). In the reverse direction, PCr is sticky and at 37 °C reacts to give creatine with approximately the same rate as it dissociates from the ternary complex; however, it dissociates from the binary complex 70–80 times faster than the net rate of creatine release (Cook et al., 1981). Thus, under equilibrium conditions the reversal phosphoryl transfer is still the rate-limiting step at pH 7. k_1 and k_{-1} are therefore

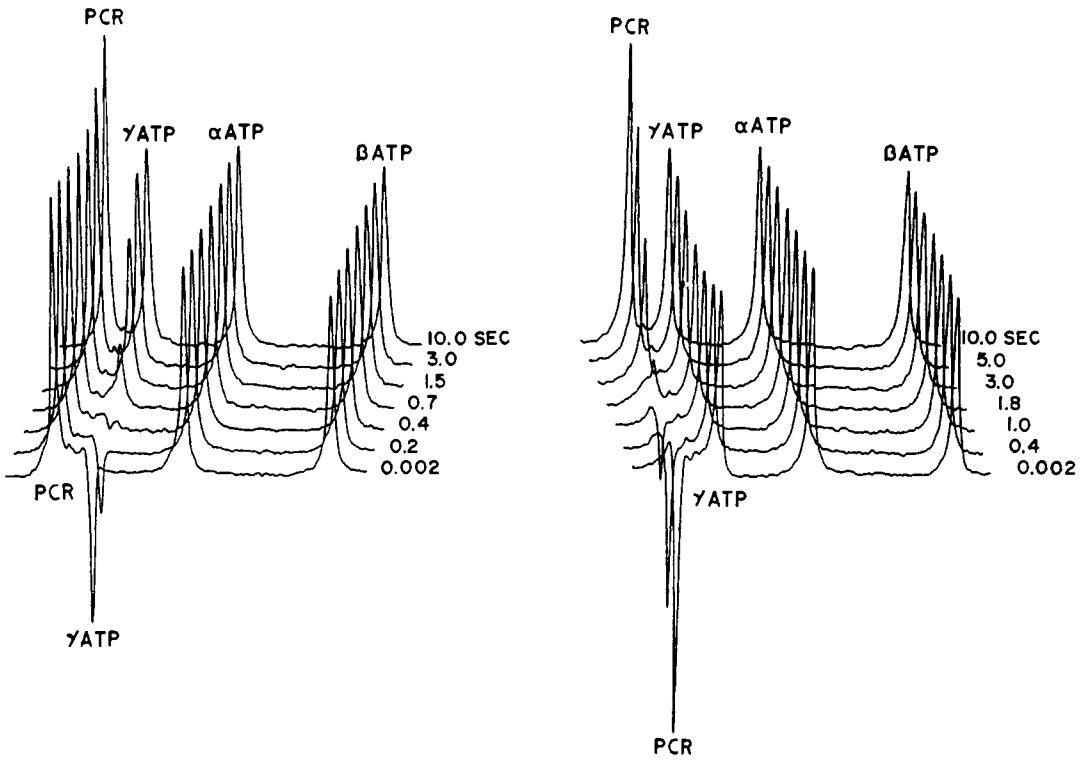

FIGURE 1: ^{31}P NMR spectra of an inversion-transfer experiment of the CK reaction in solution. The times indicate the interval between the selective 180° pulse sequence and the nonselective 90° pulse (see Materials and Methods). Eight transients were acumulated for each time point with a delay of 10 s. The solutions initially contained 50 mM Hepes, 70.0 mM KCl, 31.9 mM magnesium acetate, 30.0 mM ADP, 69.9 mM PCr, and 1000 units/mL CK at pH 6.88 and 37 °C.

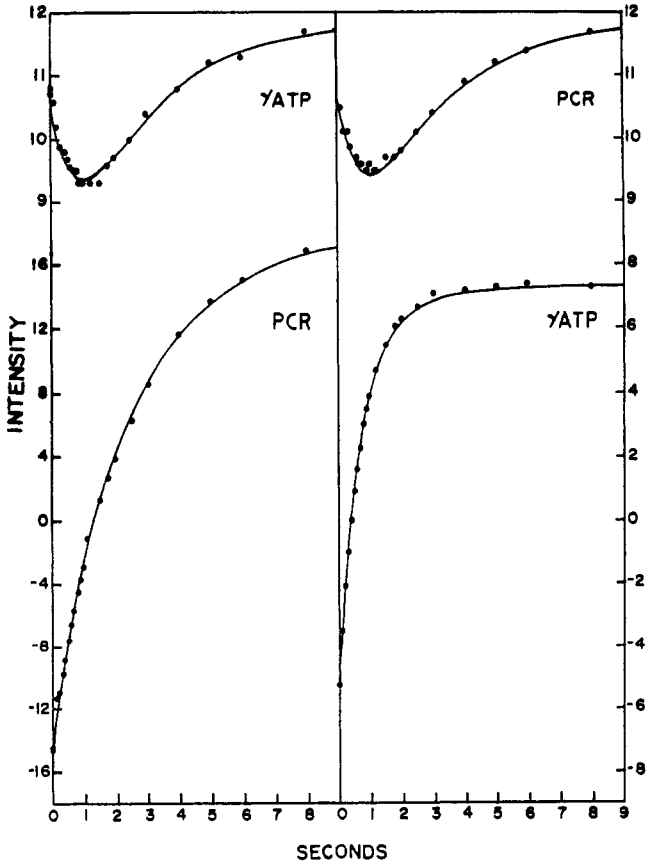

FIGURE 2: Variation with time in signal intensity of PCr and γ-ATP in an inversion-transfer experiment of the CK reaction in solution. The curves represent the best fit of the data points to eq 6 and 7, obtained as described under Materials and Methods. $M(t)$ is given in arbitrary units of intensity. Solution composition and conditions are as in Figure 1.

the rate constants for this step, which is found to be independent of pH. In kinetic studies of initial velocities of the CK reaction large changes were observed in the V_{max} and V_{max}/K_m between pH 5 and pH 10 (Cook et al., 1981). These parameters are related to the various rate constants involved in this reaction (Cook et al., 1981) including those associated with the enzyme–substrate binding processes, which are probably pH-sensitive constants (Cook et al., 1981; Rosevear et al., 1981). In our system, the ratio k_F/k_1 is constant and equals [MgADP][H] (eq 2). Under equilibrium, the variation in H^+ must therefore be countered by equal and opposite changes in MgADP. This is a direct consequence of the high apparent K_{eq}, small MgADP and H^+ concentrations, and relatively high levels of PCr, Cr, and MgATP. Similar substrate concentrations are found in perfused rat heart, so the behavior under equilibrium of the myocardial reaction kinetics with pH may be similar to that of our solutions.

Our study of the CK reaction in the Langendorff-perfused rat heart indicates that the forward and reverse fluxes through the enzyme are equal. The flux ratios calculated from the two methods are almost identical:

$$\text{flux}_F/\text{flux}_R(\text{IT}) = 0.975 \pm 0.062 \qquad n = 8$$

$$\text{flux}_F/\text{flux}_R(\text{ST}) = 0.974 \pm 0.067 \qquad n = 3$$

These results clearly indicate that the CK reaction in the Langendorff-perfused rat heart is in equilibrium, as is expected since PCr, MgATP, and pH are constant over time. There is no support for a physiological mechanism that requires substrate compartmentation in the cell.

The presence of different isozymes of CK localized in the mitochondria and myofibrils of the cardiac cell led to the proposal of the PCr "shuttle" model (Saks et al., 1978). This theory postulates that PCr and Cr function as transport molecules between pools of adenine nucleotides that have little or no diffusion through the cytoplasm. The fluxes measured

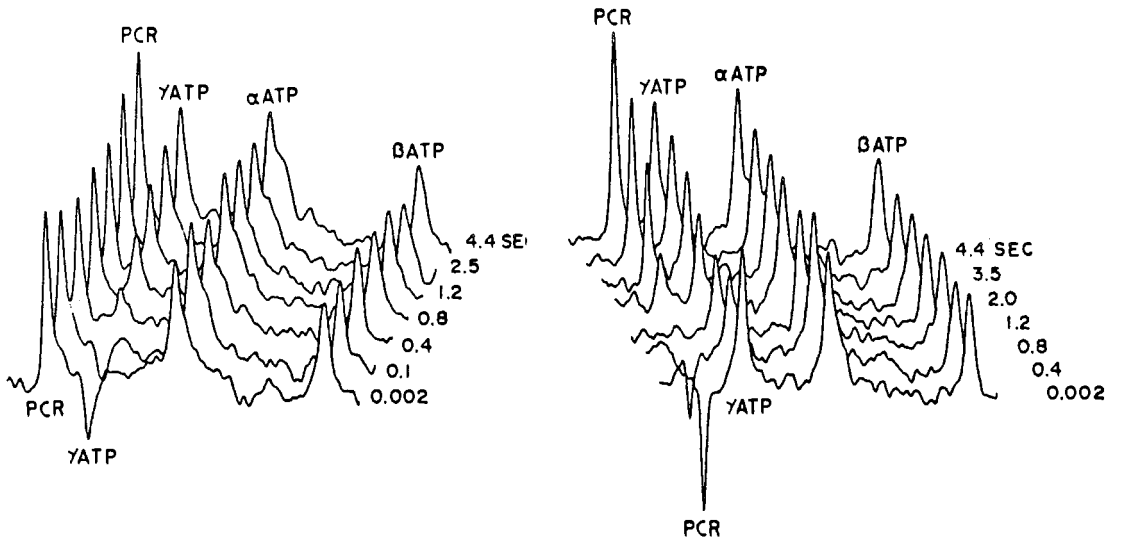

FIGURE 3: ^{31}P NMR spectra of an inversion-transfer experiment of the CK reaction in a Langendorff-perfused rat heart at 37 °C. The times indicate the interval between the selective 180° pulse sequence and the 90° pulse. A total of 48 transients was accumulated for each time point with a delay of 5 s.

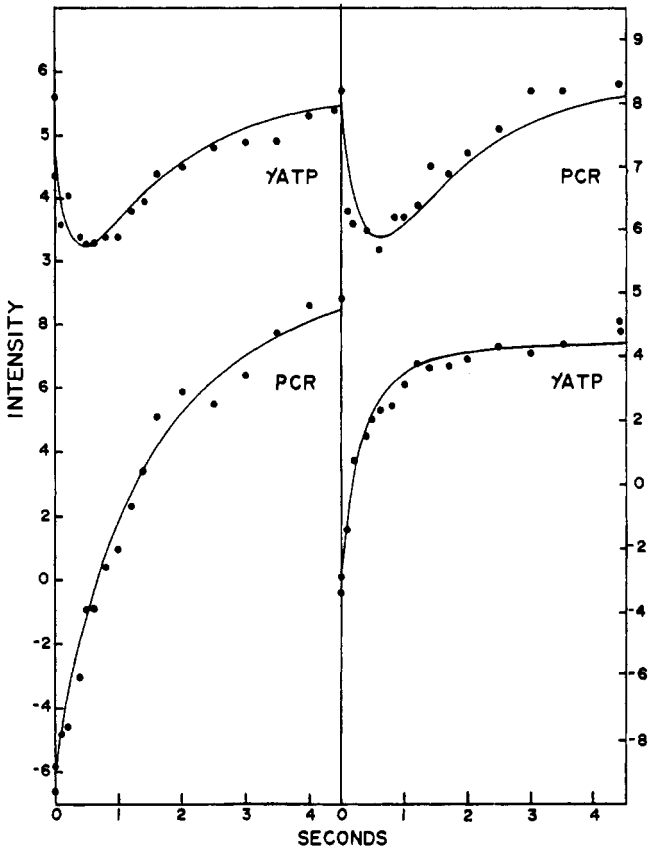

FIGURE 4: Variation with time in signal intensity of PCr and γ-ATP in an inversion-transfer experiment of the CK reaction in a Langendorff-perfused rat heart at 37 °C. The curves represent the best fit to eq 6 and 7 (Table III, heart no. 2). $M(t)$ is given in arbitrary units of intensity.

by NMR in such a model would have contributions from all the localized reactions such that $\text{flux}_F = \sum_i k_F^i[\text{PCr}]^i$ and $\text{flux}_R = \sum_i k_R^i[\text{ATP}]^i$, where each i indicates a separate pool of adenine nucleotide and CK. When PCr diffuses quickly, its average concentration is the same throughout the cell and equals $[\text{PCr}]_{\text{total}}$. The measured magnetization transfer rate constant (k_F^*) is therefore the sum of the k_Fs: $k_F^* = \sum_i k_F^i$, and the forward flux can be calculated according to $\text{flux}_F = k_F^*[\text{PCr}]_{\text{total}}$. ATP does not diffuse freely according to the shuttle model, and its concentration varies over the cell. The measured magnetization transfer rate constant k_R^* is therefore not equal to the sum of the k_rs in each compartment, and the reverse flux can be determined only if the rate constant and [ATP] are known for each compartment ($\text{flux}_R = \sum_i k_R^i[\text{ATP}]^i \neq k_R^*[\text{ATP}]_{\text{total}}$). Thus, the difference between the forward and reverse fluxes through CK reported by Nunnally & Hollis (1978) and Matthews et al. (1980) lends support to the shuttle model. Our results indicate that the fluxes calculated from the measured magnetization transfer rate constants are equal. We therefore conclude that there is no indication of compartmentation or support for the shuttle mechanism in the Langendorff-perfused rat heart. The diffusion of all CK substrates (adenine nucleotides, PCr, and Cr) between enzyme populations is faster than their interconversion rates, and the whole cell can be considered as a single compartment in terms of the CK reaction. This supports a recent observation made by Meyer et al. (1984) that because the mitochondria in rat cardiac cells are clustered densely around the small (1–2 μm) myofibrils, the sites of ATP synthesis and hydrolysis are very close together. The high-energy phosphate should therefore be able to diffuse at an adequate rate without the CK-mediated PCr shuttle.

The equality of the fluxes also indicates that other processes that utilize the ATP in the Langendorff-perfused rat heart take place at slower rates than the CK reaction and can be neglected in the calculation of the CK kinetics from inversion-transfer data.

A comparison of magnetization transfer methods allows an explanation for the differences between our results obtained by the IT and SSST methods and those from the previously published time-dependent ST (Nunnally & Hollis, 1979) and SSST (Matthews et al., 1982) experiments. The IT experiment produces data that are a function of the time following an inverting pulse during which the magnetization is allowed to relax toward equilibrium (eq 6 and 7). The analogous variable in a time-dependent saturation-transfer (TDST) experiment is the duration of the perturbation itself, and the magnetization is studied as it progresses toward a new steady state (eq 11). In the steady-state saturation-transfer experiment, only the point as time approaches infinity is taken, and the longitudinal relaxation rate $1/\tau_1$ is independently obtained (eq 12) (Matthews et al., 1982). Although IT and steady-state saturation transfer do have different constraints with respect to time and accuracy, we have shown in two different systems

that they produce the same rate constants (Tables I, IV, and V).

The limiting factor for the analysis of the saturation-transfer data (both TDST and SSST) is the measurement of the relaxation rates $1/\tau_1$ of γ-ATP and PCr. The $1/\tau_1$ obtained in an IT experiment is inherently more accurate than that from a TDST experiment because of its larger "dynamic range". An inverted peak follows a time course with an intensity range equal to almost twice the equilibrium magnetization $[2M(\infty)]$ while the TDST is usually less than $0.6M(\infty)$.

Of the several experiments available for independent determination of longitudinal relaxation rates, saturation recovery (SR) and progressive saturation recovery (PSR) [reviewed by Martin et al. (1980)] have been used in in vivo CK reaction studies (Brown & Ogawa, 1978; Matthews et al., 1982). These methods have a dynamic range of only $1M(\infty)$ and are more sensitive than IT to imperfections in pulse flip angle (Martin et al., 1980; Levy & Peat, 1975).

The accuracy of longitudinal relaxation rate data depends also on the method of analysis. Nunnally et al. (1978) in the TDST experiment obtained the T_1s from the slope of a semilogarithmic plot, which suffers from an increasingly higher statistical error in data taken at long time values. This results in a negative deviation from the true slope (Levy & Peat, 1975). Error can also result from measurement of the equilibrium intensity $M(\infty)$; deviations of 10% in $M(\infty)$ lead to a 15% error in T_1 derived from the slope of a graph of log $[M(t) - M(\infty)]$ vs. time (Martin et al., 1980).

Matthews et al. (1982) determined the apparent relaxation rates $1/\tau_1$ of γ-ATP and PCr by employing a nonselective saturation-recovery experiment with continuous selective saturation of either the γ-ATP or the PCr signal. The recovery from saturation was analyzed by using a three-parameter fit to a single exponential (Mann, 1977). However, since γ-ATP exchanges with P_i too (although with a slower rate), its T_1 recovery is biexponential, unless P_i is also saturated continuously. Thus, the above-mentioned considerations (see also Data Analysis) suggests that the $1/\tau_1$ values obtained by the IT method are more accurate.

The time constraint becomes very important in in vivo preparations, which are rarely stable over long periods of time. The relaxation and reaction rates vary from sample to sample, so it is imperative that they all be obtained from the same preparation. IT is the most time-efficient method because from a single time course the four kinetic onstants can be determined. The TDST experiment requires two independent time courses and a control spectrum for each point. This greatly extends the time required to completely characterize the kinetics of the reaction of interest by TDST. Steady-state saturation transfer (SSST) is also a time-consuming method since it requires four spectra (saturation of each peak measured at $t = \infty$ and their respective controls), plus two complete time courses for the determination of relaxation rates.

In conclusion, the fluxes through the CK reaction in the Langendorff-perfused rat heart are equal. This implies that the reaction is in equilibrium and that the diffusion of its substrates between localized pools of CK isozymes within the myocardial cell is faster than their interconversion.

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