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Measurement of an Individual Rate Constant in the Presence of Multiple Exchanges: Application to Myocardial Creatine Kinase Reaction†

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Received March 8, 1985

ABSTRACT: Forward [creatine phosphate (CP) → adenosine 5′-triphosphate (ATP)] and reverse (ATP → CP) fluxes of myocardial creatine kinase (CK) measured by using ^{31}P nuclear magnetic resonance (NMR) and conventional saturation transfer (CST) methods are unequal; this is a paradoxical result because during steady state fluxes into and out of the CP pool must be the same. These measurements, however, treat the CK reaction as a two-site exchange problem and ignore the presence of the $ATP_\gamma \rightleftarrows P_i$ exchange involving the ATPases. We have applied a method [Uğurbil, K. (1985) *J. Magn. Reson. 64*, 207] based on the saturation of multiple resonances, by which a single unidirectional rate constant can be measured unequivocally in the presence of multiple exchanges, to the measurement of CK fluxes in isovolumic rat hearts perfused under three different conditions; two of the three perfusion conditions showed a large discrepancy in the CK fluxes determined by CST, and one did not. In contrast, when the effect of the $ATP_\gamma \rightleftarrows P_i$ exchange on the CK rate measurements was eliminated, multiple saturation transfer (MST) measurements on the same hearts yielded equal forward and reverse fluxes in all cases. The rate constant for the $ATP_\gamma \rightarrow CP$ conversion measured by MST was larger than the value obtained by the conventional methodology whereas both methods gave the same rate constant in the CP → ATP direction. These results demonstrate that the cause of the paradoxical data obtained by CST measurements of CK kinetics is the $ATP_\gamma \rightleftarrows P_i$ exchange and that CK rates when determined rigorously are consistent with the CK reaction being in equilibrium. Comparison of the MST and CST data suggests that in the myocardium, a three-site $CP \rightleftarrows ATP_\gamma \rightleftarrows P_i$ scheme adequately but not fully accounts for the phosphate exchange among these compounds. The magnitudes of the CK fluxes measured by MST and CST were ~2-fold larger in hearts perfused with glucose in the absence of insulin compared to hearts where the perfusate was supplemented with pyruvate or insulin. The higher CK rate is probably the reason why the discrepancy in CK fluxes as measured by CST is not prominent in glucose-perfused hearts; this follows from the fact that for a given rate of $ATP \rightleftarrows P_i$ interconversion, the confounding influence of this exchange on the CST measurements of CK should diminish as CK fluxes increase.

Creatine kinase is present in great abundance in the myocardium, skeletal muscle, and brain; it is believed to have an important but as yet poorly understood role in bioenergetics of these tissues. Recent studies on this enzyme have utilized ^{31}P nuclear magnetic resonance (NMR)[1] spectroscopy applied to perfused organs and whole animals. In particular, the unique capability of NMR to measure reaction rates by magnetization transfer methods (Forsen & Hoffman, 1963a,b) has been exploited to examine the CK-catalyzed exchange between CP and the γ-phosphate of ATP (Brown et al., 1978; Nunnally & Hollis, 1979; Matthews et al., 1982, 1983; Seymour et al., 1983; Ingwall et al., 1983; Micelli et al., 1983; Koretsky & Weiner, 1984; Bittle & Ingwall, 1985). However, such experiments conducted on the cardiac muscle have generated a highly significant but paradoxical result; as deter-

† This research was supported by National Institutes of Health Grant R01 HL33600, Veterans Administration Medical Research Funds, and karl-Thomae GMBH (Biberach an der Riff, FRG). K.U. is the recipient of NIH RCDA Grant 1K04 HL 01241.

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; CK, creatine kinase; CP, creatine phosphate; CST, conventional saturation transfer; FID, free induction decay; LV, left ventricle; LVP, left ventricular pressure; MST, multiple saturation transfer; MVO_2, myocardial oxygen consumption rate in micromoles per minute per gram (dry weight); NMR, nuclear magnetic resonance; RPP, rate pressure product (product of heart rate and systolic pressure); SD, standard deviation; SEM, standard error of the mean; HR, heart rate; ip, intraperitoneal(ly); EDTA, ethylenediaminetetraacetic acid.

0006-2960/86/0425-0100$01.50/0

mined by conventional saturation transfer,[2] the forward[3] and reverse CK fluxes in this tissue were found to be discrepant (Brown et al., 1978; Nunnally & Hollis, 1979; Matthews et al., 1982; Ingwall et al., 1983; Koretsky & Weiner, 1984). This, however, cannot strictly be correct. CP is utilized exclusively by the CK reaction, and during steady state, its rate of formation must equal its rate of consumption. This paradoxical observation can be a consequence of a property inherent in these tissues, or it may have arisen from the well-recognized limitation of the CST method in unambiguously measuring a rate constant associated with a single reaction when the reactants participate concurrently in other chemical exchanges. Although CP is a substrate only for CK, ATP is utilized by many other enzymes, most significantly by the various ATPases; the presence of the latter activities can complicate the measurement of the rate constant associated with the ATP → CP conversion. Matthews et al. (1982) have attempted to calculate a correction for this complication using a multisite exchange model and unidirectional rate constants for the ATPase reaction derived from the MVO_2 and an ADP/O ratio of 3; they concluded that the discrepancy between the CK fluxes as determined by CST is not accounted for by ATP $\rightleftarrows$ P_i exchange. Nunnally & Hollis (1979) ascribed the discrepancy to metabolite compartmentation in the myocardium. More recently, Koretsky & Weiner (1984) provided an explanation based on the CP shuttle hypothesis, invoking the existence of exchanges catalyzed by different CK isozymes between distinct metabolite pools. However, a direct and rigorous experimental test of these explanations has not been provided. Indeed, except in a few limited circumstances [e.g., see Uğurbil et al. (1979, 1984)], it is difficult to demonstrate directly the existence of metabolite compartmentation in intact tissues. However, the measurement of a single rate constant in the presence of multiple exchanges is in principle possible (Forsen & Hoffman, 1964; Perrin & Johnston, 1979; Uğurbil, 1985). The differences among the available methods for dealing with multisite exchange problems have previously been discussed (Uğurbil, 1985). We have applied the multiple saturation transfer (MST) method (Uğurbil, 1985) to examine the CK kinetics in perfused rat hearts. The results celarly demonstrate that it is the exchange between ATP_γ and P_i that causes an underestimation of the ATP → CP flux by CST and that a three-site chemical exchange of the type CP $\rightleftarrows$ P_i adequately but not fully accounts for the kinetics of ATP_γ in the myocardium.

Materials and Methods

Langendorff Perfusion. A diagram of the Langendorff perfusion system used is shown in Figure 1. This system consisted of an approximately 120 cm long, 14 mm in diameter jacketed glass column with side ports. With the use of a small glass sinter, 95% O_2/5% CO_2 gas was bubbled up through the entire column. Prefiltered perfusate was pumped from a reservoir through a Millipore (0.45 μm) filtration capsule to the top of the column and then flowed down the column to

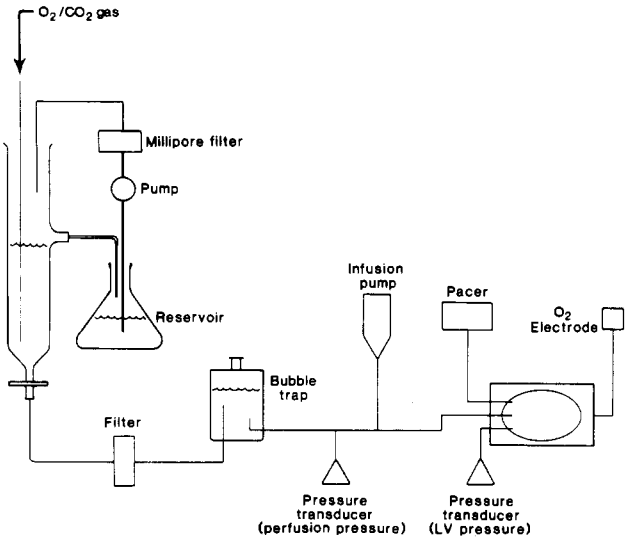


FIGURE 1: Schematic diagram of the perfusion setup.

the level of the lowest open side port from which excess perfusate drained back to the reservoir. This maintained perfusion pressure at a constant level determined by the height of the lowest open port above the heart. Perfusate flowed from the bottom of the column through a bubble chamber and then to a small heat exchanger located in the NMR probe immediately proximal to the aortic perfusion cannula. The temperatures of the heat exchanger, the bubble trap, the column, and the reservoir were regulated by using a circulating water bath. The perfusion line proximal to the last heat exchanger contained ports for pressure measurement and infusion of insulin. Previously heparinized ~400-g, Sprague-Dawley rats were anesthetized with pentobarbital (30 mg/kg, ip); subsequently, they were intubated and artificially ventilated. The heart was then removed with minimal manipulation and placed immediately in 4 °C buffer and chilled. It was then tied in place, and perfusion was begun. Care was taken not to violate the integrity of the aortic valve with the perfusion cannula. The LV was vented by using a small flanged Teflon tube. The left atrium was partially removed and a latex balloon connected to a P23DB Statham pressure transducer through a Teflon catheter inserted into the LV cavity. LVP, dp/dt, and HR were monitored through this balloon. In most cases, the latex balloon contained 75 mM P_i (pH ≃5) as an intensity reference which was used for determining the ATP and CP content of the hearts. Two small electrodes were attached to the right atrium for pacing the heart. The heart was placed in a sealed, air-free cylindrical glass chamber (18-mm i.d.) and inserted into the NMR detection coil. The sealed chamber was connected by tubing to the O_2 electrode holder. MVO_2 was determined by subracting the oxygen content of the effluent from the beating heart from the O_2 content of the perfusate entering the heart and multiplying it by the coronary flow rate. The basic perfusion medium was a slightly modified Krebs–Henseleit buffer containing 119 mM NaCl, 1.2 mM KH_2PO_4, 4.7 mM KCl, 0.1 mM EDTA, 1.2 mM $MgCl_2$, 28 mM $NaHCO_3$, and 1.8 mM $CaCl_2$. The carbon source was different for the different sets of measurements as described below. In all cases, perfusion pressure was 85 mmHg, and the end diastolic pressure was ~8 mmHg by adjusting the volume of the latex balloon in the LV. The coronary flow averaged over all the hearts was 20 ± 2 (SD) mL/min. It should be noted that because of the vent, LV accumulation of perfusate (via the thebesian circulation) could not occur, and the aortic valve remains closed during contraction. Under

[2] We will refer to the saturation transfer measurements based on two-site exchange kinetics as conventional.

[3] Unidirectional fluxes of CK in the forward and reverse directions refer to the rate of conversion of CP to ATP and of ATP to CP, respectively. The terms "flux" and "rate" have been used interchangeably for the same parameter in the past; this usage is adapted here as well. For a given reaction, if the two unidirectional fluxes or rates are not equal, as is the case when nonequilibrium conditions prevail, the difference between the two unidirectional fluxes or rates will be referred to as the *net rate* for that reaction. Note that unidirectional rates are always equal to or larger than the *net* rates.

these circumstances, the balloon constitutes a nonvarying preload as well as the afterload against which the ventricle contracts.

The kinetic measurements were performed on hearts perfused under three conditions which differed in the carbon substrate, pacing rate, and temperature of the heart. These conditions were the following: (I) the substrate was pyruvate plus glucose (10 mM each), the hearts were paced at 390 beats/min, and the perfusion temperature was 38 °C; (II) the substrate was glucose in the presence of 5 IU/L insulin, the hearts were paced at 360 beats/min, and the perfusion temperature was 38 °C; (III) the substrate was glucose without insulin, the heart rate was 360 beats/min, and the perfusion temperature was 35 °C. These three conditions were chosen so as to obtain different levels of MVO_2 and different degrees of discrepancy between the forward and reverse fluxes of CK.

NMR Measurements. ^{31}P NMR measurements were conducted at 146.1 MHz with a single-turn solenoidal probe of our design and manufacture. CST measurements were performed by saturating either ATP_γ or CP to measure the CK rate constant associated with the forward direction or the reverse direction, respectively. MST measurements were performed by first irradiating the P_i resonance, establishing a new steady state for the resonance intensities, and subsequently conducting a measurement just as in CST while retaining the P_i saturation. Thus, in MST, the fractional reductions were determined from resonance intensities measured while saturating P_i only, and while saturating both P_i and one other resonance (either CP or ATP_γ); the spin relaxation rates were determined under the latter saturation conditions.

One of the two frequencies at 146.1 MHz used for selective saturation of the ^{31}P resonances was derived from the same source that is normally used to generate the nonselective high-power pulses; a computer-controlled fast switch shunted the radio frequency from this channel either through a high-power pulse amplifier for the nonselective hard pulses or through a low-power amplifier for the selective irradiation. The output from this channel (which we will refer to as f1) was in the continuous-wave mode during low-power selective irradiation and was turned off during FID acquisition. The second selective irradiation frequency (f2) was generated by using a separate, computer-controlled frequency synthesizer and an amplifier. This channel was gated so that the output was in the form of 12-μs square pulses at 60-μs intervals. The two frequency sources were combined through a 10-dB directional coupler, passed through a 146-MHz band-pass filter, and then channeled through the normal circuitry of the spectrometer into the probe. f1 and f2 were turned on for both the CST and MST measurements. f2 was used to saturate the ATP_γ or the CP peaks; f1 was used to saturate the P_i resonances during the MST measurements. During the CST procedure, f1 was moved 1100 Hz upfield of the ATP_γ resonance so that the frequency separation between f1 and ATP_γ was equal to the frequency separation between P_i and ATP_γ. This procedure assured that any indirect, off-resonance saturation effects not mediated by chemical exchange were the same on the ATP_γ resonance in both the MST and CST measurements; this allowed us to compare the fractional reduction and the T_1's of the ATP_γ peak between the MST and CST measurements. Even though such a control did not exist for the CP resonance, the off-resonance effect on the CP intensity of irradiating the P_i peak was ~10%.

In hearts perfused with P_i-containing medium, two P_i peaks arising from the extracellular and cytosolic pools (P_i^{ex} and P_i^{cy}, respectively) are detected. In addition, a mitochondrial P_i resonance appearing at the same approximate position as extracellular P_i has previously been identified (Garlick et al., 1983). In the MST experiments, f1 was positioned approximately halfway between P_i^{ex} and P_i^{cy} resonances, and the power was adjusted so as to saturate all the P_i resonances.

Except for the position of the f1 frequency, the procedure was identical for both the MST and CST measurements. To obtain a single determination of the two CK rate constants associated with the forward and reverse directions (k_f and k_r, respectively) either by the CST or by the MST procedure, 20 spectra were recorded using 90° pulses and a list of different repetition times. FID's were acquired by cycling through this list 4 times, summing 12 FID's for each spectrum during each cycle. Out of the 20 files collected, the first 9 were for determining k_f and the remainder for k_r. Within the nine spectra recorded for calculating k_f, one was obtained with a 12-s repetition time (allowing full relaxation for the CP peak), with f2 positioned downfield from CP symmetrically opposite from ATP_γ; the remaining eight were acquired while saturating ATP_γ with repetition times ranging from 0.4 to 4.1 s; these eight spectra constitute a progressive saturation sequence. The CP intensities obtained from them were analyzed by using a nonlinear, least-squares, two-parameter fit for T_1 and the fully relaxed intensity of the CP spins while ATP_γ is saturated. A similar sequence was followed in the 11 spectra recorded for calculating k_r except that 9 out of 11 were obtained by using varying repetition times ranging from 0.2 to 2.2 while f2 was irradiating the CP resonance and 2 were retecorded with a 5-s repetition time while f2 was positioned upfield of the ATP_γ resonance symmetrically opposite from the CP peak (see Figure 4). Since the nonselective T_1 of ATP_γ is ~1 s (in the absence of saturating any other resonance), 5 s was sufficient to allow full relaxation of this peak. During CST, the f1 frequency was set at a control position as described before. In the MST procedure, f1 was used to saturate the P_i resonances continuously during the acquisition of all 20 spectra. The time required for acquiring the 20 spectra, total of 48 scans each, was ~35 min. At least four but typically six such measurements were performed on each heart alternating between CST and MST saturation patterns; thus, two or three measurements of CST and MST each were obtained on the same heart. Approximately 85% of the time, the hearts were stable both in LVP and in ATP, CP, and P_i content to allow this. If they showed signs of early deterioration in mechanical output or in the CP, ATP, and P_i content, the measurements were discontinued, and the data already acquired were disregarded. For each heart, a fully relaxed spectrum was recorded with both f1 and f2 turned off before and after the kinetic measurements; these spectra were used to calculate the ATP, CP, and P_i content of these hearts using integrated intensities and the resonance from the 75 mM acidic P_i solution contained in the latex balloon.

RESULTS

Properties of the Perfused Hearts. Table I lists some of the mechanical and biochemical properties of the hearts perfused under the three different conditions. Typical spectra obtained from these hearts are shown in Figure 2. The ATP content of these hearts under conditions I and II appears invariant and is similar to values reported previously (Ingwall, 1982). Even though the myocardial ATP content under perfusion condition III was not specifically determined, comparison of ^{31}P NMR spectra (Figure 2) suggests that it is comparable to the values obtained for conditions I and II.

The three perfusion conditions employed in these experiments were chosen in order to establish states that accrued

Table I: Properties of Perfused Rat Hearts under the Three Different Perfusion Conditions Used[a]

perfusion condn	N^b	gram dry weight	ATP (μmol/g dry weight)	CP/ATP ratio	MVO_2 [μmol (g dry weight)$^{-1}$ min^{-1}]	RPP[c] (mmHg/min)
I	8	0.26 ± 0.02/0.05	27.4 ± 2.4/6.4	2.08 ± 0.05/0.13	46.3 ± 2.2/6.2	46580 ± 3769/10660
II	6	0.28 ± 0.01/0.02	26.1 ± 1.8/4.5	1.57 ± 0.03/0.06	41.2 ± 2.5/7.0	40980 ± 1938/4746
III	4	0.26 ± 0.01/0.02	ND[d]	1.07 ± 0.08/0.15	29.1 ± 2.3/4.6	31076 ± 1965/3930

[a] All values are mean ± SEM/SD. Langendorff-perfused isovolumic rat hearts paced at 390 beats/min when the perfusion substrate was pyruvate + glucose (I) and at 360 beats/min when the substrate was glucose with (II) or without (III) insulin. The end diastolic pressure was set at 8 mmHg. See Materials and Methods for further details. [b] Number of hearts on which the parameters listed were determined. [c] Systolic pressures were 119 ± 26/9, 114 ± 13/5, and 86 ± 11/5.5 mmHg for conditions I, II, and III, respectively. RPP is simply the product of the systolic pressure and the heart rate which was fixed by pacing. dp/dt, which was also determined for these hearts, was 4340 ± 1240/438, 4722 ± 763/311, and 3835 ± 336/168 mmHg/s for conditions I, II, and III, respectively. [d] Not determined.

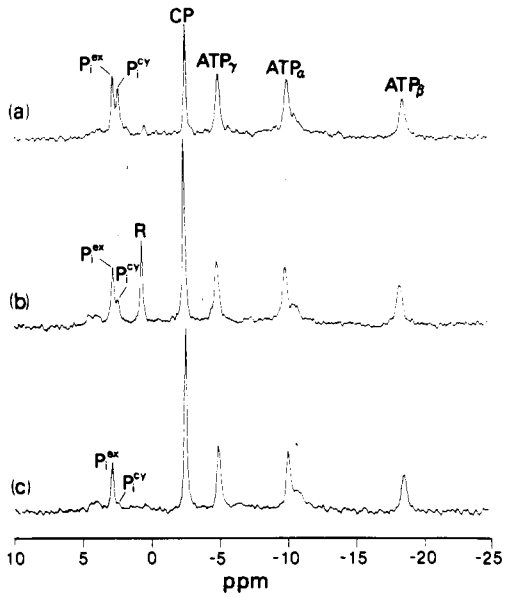


FIGURE 2: ^{31}P NMR spectra at 146.1 MHz of perfused rat hearts under perfusion conditions I, II, and III (a, b, and c, respectively). 32 FID's were obtained with 90° pulses and a 12-s repetition time. Peak R is the phosphate solution (pH ~5) present in the latex balloon in this heart.

in different degrees of discrepancies in the CST determinations of the myocardial CK flux. Previously published data (Matthews et al., 1982; Ingwall et al., 1983, 1984; Micelli et al., 1983) suggested to us that the discrepancy in the CK fluxes may be most pronounced under conditions when ATP synthesis and utilization rates are high (i.e., MVO_2 and RPP are high) yet the heart is able to maintain a high CP/ATP ratio. The conclusions reached in this paper provide a justification for this generalization and will be discussed in greater detail later. The three perfusion conditions employed in this work provided us with two states (I and II) of high MVO_2, RPP, and CP/ATP ratios and a relatively high discrepancy in CK fluxes, and one state (III) with a lower MVO_2 and RPP, a CP/ATP ratio of ~1, and virtually no discrepancy. For the hearts to operate at high RPP's while maintaining a high CP/ATP ratio, inclusion of either pyruvate or insulin in the perfusate was necessary. Hearts perfused with glucose alone were simply not able to maintain a CP/ATP ratio much larger than ~1 even at RPP's much lower than those used here. When insulin was included at commonly used doses (~20 IU/L), the CP/ATP ratio increased to approximately 1.7–2, and the hearts were very similar to those perfused with pyruvate plus glucose. Therefore, the insulin dose was lowered to obtain a CP/ATP ratio intermediate between the values observed under conditions I and III.

Our only interest in state III was to perform MST measurements under conditions where CST gave no discrepancy. Thus, it served as a control for MST; in this capacity, it was very satisfactory even though the temperature was lower than that used under conditions I and II. At 38 °C, hearts perfused with glucose in the absence of insulin, and operating at ~30000 RPP, the CK rate constants k_f and k_r were ~1 s^{-1}. In consequence, a large reduction in the CP intensity occurred when ATP_γ was saturated; this effect, combined with the fact that the CP content of glucose-perfused hearts is relatively low, rendered the measurement of k_f more difficult and error prone than it was under conditions I and II. Therefore, the temperature was lowered in order to reduce the CK rate and thereby improve the accuracy of each individual measurement considerably.

Measurement of Rate Constants. CST has previously been used to measure rate constants and will not be described in detail here. It is a method which is correct for a two-site exchange problem. It can be executed in several different ways. The procedure used here is described in detail under Materials and Methods.

If $ATP_\gamma \rightleftarrows P_i$ exchange in the myocardium is not ignored, to a first approximation the γ-phosphate of ATP exchanges according to

$$CP \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ATP_\gamma \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} P_i$$

where we have labeled the rate constants for the $ATP_\gamma \rightleftarrows P_i$ exchange as k_a and k_{-a}. We have avoided using the notation k_h and k_s for ATP "hydrolysis" and "synthesis" because, as discussed further on, it is not possible at this stage to specifically identify these rates as such. In a reaction scheme of this type, the correct k_r and k_f can be determined by the MST procedure which has been described in detail previously (Uğurbil, 1985); the procedure is equivalent to performing a CST-type measurement while retaining the P_i spins continuously saturated. In this case, k_r is given by the equation:

$$k_r = \frac{\alpha_\gamma}{\sigma}\left(\frac{M_\gamma^* - M_\gamma^{**}}{M_\gamma^*}\right)$$

where M_γ^* and M_γ^{**} are the steady-state magnetizations of ATP_γ spins while only P_i is saturated and while both CP and P_i are saturated, respectively. σ is a parameter defined by the equality $M_\gamma^\circ/M_{cp}^\circ = \sigma(M_\gamma^*/M_{cp}^*) = k_f/k_r$ where the superscript o denotes Boltzmann thermal equilibrium magnetizations. σ is easily measured by comparing spectra recorded with and without saturating the P_i spins. α_γ is equal to $T_{1\gamma}^{-1} + k_r + k_a$ were $T_{1\gamma}^{-1}$ is the spin–lattice relaxation rate of ATP_γ in the *absence* of any exchange; α_γ is the spin–lattice relaxation rate of ATP_γ while both CP and P_i are saturated. By saturation of ATP_γ while retaining the saturation of P_i spins, k_f can also be determined by the MST method (Uğurbil, 1985). However, because CP exchanges only with ATP_γ, k_f can be rigorously determined by CST as well.

Table II: Forward and Reverse Rate Constants and Their Ratios and the Ratio of the Forward and Reverse Fluxes for Myocardial CK in Perfused Rat Hearts[a]

perfusion condn[b]	method of measurement	N[c]	k_f (s^{-1})	k_r (s^{-1})	k_r/k_f	F_r/F_f
I	CST	24	0.34 ± 0.02/0.09	0.39 ± 0.03/0.15	1.18 ± 0.10/0.51	0.56 ± 0.04/0.17
	MST	24	0.30 ± 0.02/0.08	0.61 ± 0.04/0.18	2.13 ± 0.16/0.76	1.03 ± 0.07/0.36
II	CST	15	0.51 ± 0.02/0.06	0.49 ± 0.04/0.14	0.95 ± 0.07/0.26	0.62 ± 0.05/0.17
	MST	15	0.46 ± 0.02/0.06	0.64 ± 0.04/0.16	1.41 ± 0.09/0.35	0.92 ± 0.06/0.23
III	CST	8	0.64 ± 0.04/0.11	0.61 ± 0.04/0.12	0.98 ± 0.08/0.23	0.93 ± 0.10/0.28
	MST	8	0.74 ± 0.07/0.08	0.69 ± 0.03/0.09	0.97 ± 0.08/0.22	0.94 ± 0.08/0.23

[a] All reported values are mean ± SEM/SD. [b] See Materials and Methods. [c] N is the number of individual measurements of k_r and k_f; the number of hearts used for these measurements was eight hearts for conditions I, six for II, and four for III.

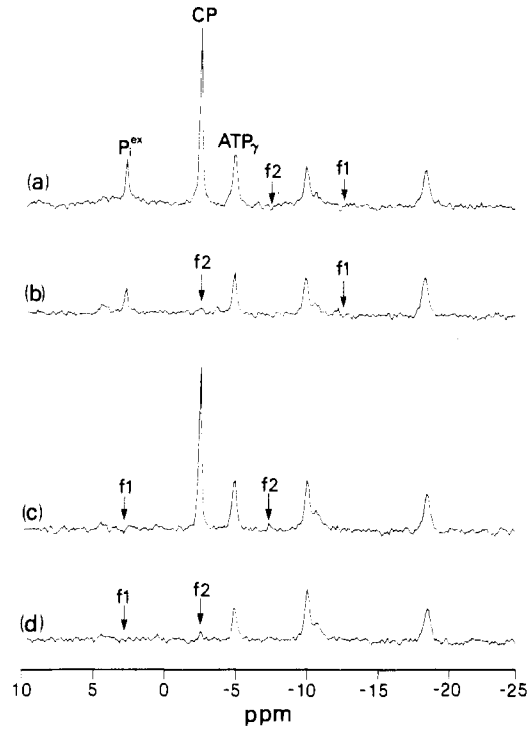


FIGURE 3: ^{31}P spectra at 146.1 MHz from CST (a and b) and MST (c and d) measurements of the CK reaction rate constant in the ATP → CP direction. Arrows designate the position of the selective irradiations f1 and f2. All spectra are the sum of 48 FID's recorded with 90° pulses; the repetition time was 5 s for (a) and (c) and 2.2 s for (b) and (d); it should be remembered that the spin–lattice relaxation of ATP_γ is ~1 s in (a) and (c) but ~0.5 s in (b) and (d) where CP is saturated.

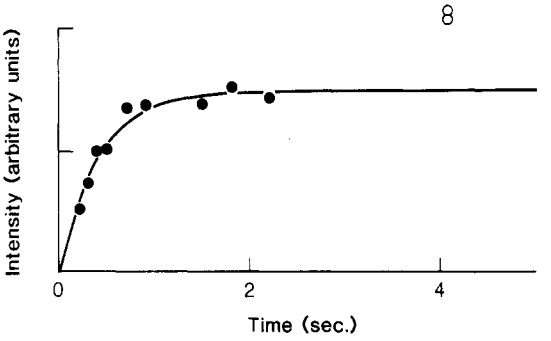


FIGURE 4: Example of the data points collected during measurement of k_r by MST. (●) ATP_γ peak heights obtained at different repetition times while both CP and P_i were saturated; the solid line is a two-parameter (T_1 and fully relaxed intensity) nonlinear least-squares fit to these data. (○) Two measurements of ATP_γ peak heights when only the P_i resonance was saturated by f1, and f2 moved to the position shown in Figure 3c.

Two spectra each from the CST and MST measurements of k_r in hearts perfused under condition I are illustrated in Figure 3. During either measurement, f2 either saturated the CP resonance (Figure 3b,d) or was at a control position upfield of ATP_γ symmetrically opposite from the CP resonance (Figure 3a,c). A progressive saturation T_1 measurement was performed under the conditions of Figure 3b,d. From the ATP_γ intensity in Figure 3a,c (which was obtained with sufficiently long repetition times to allow full relaxation) and the fully relaxed intensity of ATP_γ obtained from the fit to the progressive saturation T_1 data, the fractional reduction in the ATP_γ intensity induced by saturation of the CP resonance was calculated. An example of this type of data for k_r determination is shown in Figure 4. A similar set of measurements with f2 saturating ATP_γ was used to obtain k_f. From spectra recorded with and without saturation of P_i, σ was determined to be between 1 and ~1.1 for the three perfusion conditions. The reason for this is that saturating P_i caused only a small effect on ATP_γ which was detectable (~10% reduction) only under perfusion condition I and a substantial fraction of any effect on ATP_γ shows up on the CP resonance due to the relatively rapid CK rates. For all the calculations, σ was taken as 1.

Table II shows the rate constants, the ratio of the rate constants, and the ratio of the forward and reverse fluxes (F_f and F_r, respectively) for the CK reaction obtained by both MST and CST. F_r/F_f is simply equal to $(k_r/k_f)([ATP]/[CP])$. k_r, k_f, k_r/k_f, and F_r/F_f were calculated from each individual measurement and were then averaged to obtain the mean, the SD, and the SEM. From the MST data (Table II), and ATP and CP contents (Table I), the CK flux in both the reverse and forward directions was calculated to be 16.7 μmol s^{-1} (g dry weight)$^{-1}$ under conditions I and II; this is equivalent to ~8.4 mM/s, given the water content of the myocardium per gram dry weight (Morgan et al., 1964). Under perfusion conditions III, assuming that the myocardial ATP level is the same as in the other two conditions, the CK flux was also ~17 μmol s^{-1} (g dry weight)$^{-1}$. However, the temperature in these hearts was relatively low (35 °C, see Materials and Methods); when the temperature was raised to 38 °C while all other parameters including the RPP were kept constant, k_f and k_r were ~1 s^{-1} which, using a value of 27 μmol of ATP/g dry weight, gave a CK flux of ~27 μmol s^{-1} (g dry weight)$^{-1}$, or ~14 mM/s. This value is very similar to that reported previously (Ingwall et al., 1984; Bittle & Ingwall, 1985) under virtually identical perfusion conditions.[4]

Table III gives the fractional reductions and the effective T_1's obtained during the MST and CST measurements of k_r. The large standard deviations associated with the numbers shown in Table III predominantly arise from the variation from

[4] These investigators reported a k_f value of 1.30 at the specified RPP and MVO_2; a CK forward flux of ~35 μmol (g dry weight)$^{-1}$ s^{-1} is calculated from this number by using a CP/ATP ratio of ~1 under these perfusion conditions and an ATP level of 27 μmol/g dry weight which, as discussed before, appears to be relatively invariant in the well-oxygenated, isolated perfused hearts.

Table III: Fractional Reduction and T_1 of the ATP_γ Resonance in MST and CST Measurements[a]

perfusion condn	method	N	ATP_γ fractional reduction[b]	ATP_γ T_1 (s)[c]
I	CST	24	0.20 ± 0.01/0.07	0.55 ± 0.02/0.07
	MST	24	0.26 ± 0.01/0.05	0.45 ± 0.01/0.05
II	CST	15	0.24 ± 0.01/0.05	0.50 ± 0.02/0.09
	MST	15	0.25 ± 0.01/0.04	0.40 ± 0.02/0.08
III	CST	7	0.24 ± 0.01/0.02	0.42 ± 0.03/0.07
	MST	7	0.26 ± 0.01/0.02	0.38 ± 0.02/0.04

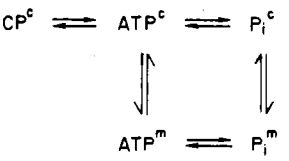
[a] All values are mean ± SEM/SD. T_1 values were measured while saturating CP only in the CST measurements and while saturating both CP and P_i during the MST measurements. [b] Fractional reduction is $(M^* - M^{**})/M^*$ for MST and $(M^0 - M')/M^0$ for CST where M^0 is the Boltzmann thermal equilibrium magnetization, M^* is the steady-state magnetization established subsequent to saturating P_i, and M' and M^{**} are the steady-state intensities established when CP and CP + P_i are saturated, respectively. [c] Measured while saturating the CP resonance during CST experiments and while saturating both CP and P_i resonances during MST experiments.

one heart to another. The intrinsic random errors associated with a single determination of either the fractional reduction or the T_1 were comparable to or less than the SD values reported for the means (Table III). The fractional reductions and intrinsic T_1's are correlated; for the same flux, different T_1's will lead to different fractional reductions. A similar correlation exists between the rate constants and the CP and ATP contents; for a given flux, higher levels of CP and ATP mean lower k_f and k_r, respectively. This is simply a consequence of the fact that the pseudo-first-order rate constants determined are the inverse of the lifetimes of these metabolites due to the CK activity. Because of these correlations and the variations from one heart to another, the rate constants and flux ratios should be calculated individually for each measurement and subsequently subjected to an averaging and error analysis as they were done for Table II. Thus, the appropriate errors associated with the magnitude of the rate constants and flux ratios are those given in Table II and not those calculated from the standard deviations supplied in Table III.

Under conditions I and II, the *net* rates of ATP synthesis[5] due to oxidative phosphorylation were 4.6 and 4.1 μmol s^{-1} (g dry weight)$^{-1}$, respectively, as calculated from the MVO_2 (Table I) using a ADP/O ratio of 3. These are only a factor of ~3.5–4 lower than the CK fluxes. Although it is highly desirable to know the *unidirectional* $ATP_\gamma \rightleftarrows P_i$ exchange rates for comparison with the CK fluxes, it was difficult or impossible to obtain them by using saturation transfer. Under conditions I and II (Figure 2), it was not possible to measure a rate in the $P_i \rightarrow ATP_\gamma$ direction by saturating ATP_γ spins and observing its effect on the P_i resonances because the intracellular P_i peaks (Figure 2) were very small. The determination of the rate in the $ATP_\gamma \rightarrow P_i$ direction by NMR is, in general, difficult because of the presence of the relatively rapid $CP \rightleftarrows ATP_\gamma$ exchange; even though the complication introduced by the CK reaction can be eliminated by using

[5] The net ATP synthesis rate is calculated from the formula $6MVO_2/60$ where the factor 6 is the number of ATP molecules synthesized per O_2 consumed (ADP/O ratio of 3) and factor 60 is to convert to units of s^{-1}. During the steady state, this rate must equal the net ATP consumption rate. Note that the net rate may be different from the unidirectional rates of the various ATPases. For example, the mitochondrial ATPase may have a high rate in both ATP synthesis and hydrolysis directions; however, during oxidative phosphorylation, the rate of this particular enzyme in the synthesis direction must exceed its rate in the hydrolysis direction. The difference between these two unidirectional rates will be the *net* rate of ATP synthesis.

Scheme I



MST, the fractional reductions to be measured remain no larger than that observed in a conventional experiment where P_i is saturated alone (Uğurbil, 1985).

Discussion

The data in Table II demonstrate that under perfusion conditions I and II, there is a large discrepancy between the forward and reverse CK fluxes as determined by CST. This discrepancy disappears, and the F_r/F_f ratio attains the theoretically expected value of 1 when the CK rate constants are measured by the MST procedure in which the potential effect of the $ATP \rightleftarrows P_i$ exchange is eliminated. The correction comes as a result of higher k_r values obtained by the MST procedure. Within the error of the measurements, k_f values as determined by CST and MST are the same. This indeed should be the case if the failure of CST to measure the correct fluxes arises from $ATP \rightleftarrows P_i$ exchange. Experiments performed under perfusion condition III serve as a control; it is seen that when fluxes obtained by CST are not discrepant, the CK rate constants measured by MST and CST are virtually the same. Consequently, we conclude that the discrepancy in the CK fluxes as determined by CST arises due to the multiple exchanges involving ATP_γ, specifically the ATPase-catalyzed $ATP_\gamma \rightleftarrows P_i$ exchange. This is in direct opposition to the conclusion reached by Matthews et al. (1982), who have attempted to calculate the effect of the $ATP \rightleftarrows P_i$ exchange on the CST determination of CK rates; although these investigators used a three-site exchange model in calculating their fractional reductions, their T_1 values for the ATP_γ resonance were derived by assuming a two-site exchange between ATP_γ and CP.

In the intact cell, there are additional reactions besides the $ATP \rightleftarrows P_i$ exchange that utilize ATP. In particular, the activity of several glycolytic enzymes may be considered as a possible source of complication in CST measurements. however, even under conditions II, the glycolytic flux is expected to be too slow to influence CK rate determinations by CST. This follows from the fact that the *maximal* glucose utilization rate by isolated rat hearts was measured to be ~16 μmol (g dry weight)$^{-1}$ min^{-1} (Kobayashi & Neeley, 1979).

Given the primary conclusion reached from these experiments, it is in principle possible to quantitatively account for the discrepancy observed in the CST measurements. In practice, however, this is very difficult. In the context of a three-site exchange among CP, ATP_γ, and P_i, the ATP_γ T_1 measured in the MST experiment (Table III) has an easily identifiable physical meaning; it is equal to $(T_{1\gamma}^{-1} + k_r + k_a)^{-1}$. However, the ATP_γ spin–lattice relaxation obtained while saturating only CP should be a double exponential. Because it is extremely difficult to resolve the two exponentials, a single exponential is used to fit the data. The number obtained from this fit, then, is an average of the two exponential time constants. Therefore, a quantitative comparison of the two spin–lattice relaxation times obtained by the MST and CST methods is not possible; however, it is reasonable to expect that the T_1's obtained by MST should be shorter if the $ATP_\gamma \rightleftarrows P_i$ exchange is significant. According to a nonpaired t-test analysis, they were significantly shorter ($p < 0.001$) under conditions I and II only (Table III).

From the point of view of spin dynamics, at the very least a three-site exchange of the type CP $\rightleftarrows$ ATP_γ $\rightleftarrows$ P_i must be considered as an operative kinetic model in order to account for the MST results. However, this scheme is at best an approximation, because ATP_γ $\rightleftarrows$ P_i exchange is a complicated reaction mediated through subcellular compartments. Taking into account the mitochondrial/cytoplasmic compartmentation of ATP, one needs to consider a general kinetic scheme of the type shown in Scheme I where superscripts m and c denote mitochondrial and cytoplasmic, respectively. Whether P_i^c $\rightleftarrows$ P_i^m exchange is fast or not is immaterial for our experiment, since all P_i resonances (cytoplasmic, mitochondrial, and extracellular) appear in close proximity to each other in the ^{31}P NMR spectrum and are all saturated during the MST measurement. The first question that arises in considering a scheme of this type is the origin of the ATP resonances detected in the ^{31}P NMR spectra of hearts. There is no direct data to answer this question rigorously. However, as much as ~20% of the intracellular ATP in the myocardium is thought to be mitochondrial (Illingworth et al., 1975). ATP_γ resonances observed in intact heart spectra are ~30 Hz wide at half-height. On the other hand, studies with isolated mitochondria have shown that phosphorus resonances detected from mitochondrial ATP are very broad (~100–150 Hz full width at half-maximum for ATP_γ) (Ogawa et al., 1978; Ogawa & Lee, 1982). The difference in line widths implies that only ~4–7% of the ATP_γ resonance peak height comes from mitochondrial ATP; this, however, is not better than the signal to noise ratio of the NMR spectra. Since all kinetic measurements were performed with peak heights, we suggest that the ATP pool monitored in these measurements is predominantly cytoplasmic. All subsequent discussion will be based on this premise.

Within the context of Scheme I, two possible exchange conditions need to be considered. The first is that the mitochondrial and cytosolic ATPs are in slow or intermediate exchange relative to their T_1's. In this case, one has to consider the ATP^m $\rightleftarrows$ ATP^c reaction as one of the multiple exchanges that causes problems in the CST measurement of the CK rates; the elimination of this complication in the MST experiments conducted requires that the saturation of the P_i resonances lead to the saturation of ATP^m. This can occur if the translocase is working predominantly in the direction of transferring ATP^m to the cytoplasm, and the lifetime of ATP^m (which is determined by the $ATP^m \rightarrow ATP^c$ and $ATP^m \rightarrow P_i^m$ reactions) is short relative to the intrinsic[6] ATP_γ^m T_1. The second possibility is that ATP^m and ATP^c are in fast exchange relative to their T_1's. We favor the former condition because studies on the mitochondrial ATP–ADP translocase indicate that this transport system in *energized* mitochondria works predominantly in the direction of ATP extrusion from the mitochondria (Klinberg et al., 1976).

The CP $\rightleftarrows$ ATP_γ $\rightleftarrows$ P_i scheme, however, does not strictly explain all the data. In particular, the fractional reductions in the ATP_γ resonance observed in the MST and CST procedures under perfusion condition I only are inconsistent with this scheme; in the limit $\sigma \sim 1$, for an equilibrium exchange kinetics of the type CP $\rightleftarrows$ ATP_γ $\rightleftarrows$ P_i, the fractional reductions observed in the ATP_γ resonance by the MST procedure should be equal to or less than the value observed by CST (Uğurbil, 1985). This is indeed the case under conditions II and III but not condition I (Table III). The difference is small, but given the number of measurements performed, it was statistically significant ($p < 0.001$). One possible explanation is that there is a small but net flux through the CK reaction monitored by these measurements (i.e., the forward and reverse fluxes are not strictly equal). A reaction scheme of the type shown in Scheme II can lead to the fractional reductions observed under condition I. In a reaction scheme like this, the *apparent* CK "forward" rate constant that would be measured by either the MST or the CST procedure would in fact be $k_d + k_f$; steady-state conditions would require that $(k_d + k_f)[CP] = k_r[ATP]$. Thus, if Scheme II is valid, the *apparent* fluxes calculated from the saturation transfer experiments should still be equal as they are in the MST measurements. Within the context of Scheme II, it is possible to provide an explanation for the fact that the deviation from the CP $\rightleftarrows$ ATP_γ $\rightleftarrows$ P_i model occurs only under condition I; if k_d is a constant (such as a diffusional constant) which does not depend on perfusion conditions, then as k_f gets smaller (Table II), the fractional reduction of ATP_γ between MST and CST measurements would become more pronounced.

Scheme II

$$\xleftarrow[k_d]{} CP \underset{k_r}{\overset{k_f}{\rightleftarrows}} ATP_\gamma \rightleftarrows P_i \xleftarrow{}$$

A better understanding of the phosphate exchange kinetics among CP, ATP, and P_i requires a knowledge of rates associated with the ATP $\rightleftarrows$ P_i exchange. Direct measurements of these unidirectional rates were impractical as mentioned under Results. It has previously been reported that a rate obtained by saturating the γ-phosphate resonance of ATP (presumably both cytosolic and mitochondrial) and observing the effects on *cytosolic* P_i is related to MVO_2 by a P/O ratio of 3.5 ± 0.8 (SEM) (Matthews et al., 1981). However, saturation transfer measurements of this type are difficult to interpret because of the involvement of both the mitochondrial ATPase and P_i transport steps in the effect that is being monitored (see Scheme I). Therefore, it is difficult to quantitatively assess from MVO_2 the unidirectional rate constants associated with ATP $\rightleftarrows$ P_i exchange that are relevant for the CST measurements of CK kinetics. Considering the problem qualitatively, however, it is reasonable to expect that, as the mechanical output and the MVO_2 increase, both the net rate of ATP synthesis and the unidirectional rates involved in the process increase. This in turn should result in a more prominent effect on the CST determination of CK fluxes. It is important to note that the CK rate is also coupled to MVO_2 (Ingwall et al., 1983, 1984; Bittle & Ingwall, 1985; Micelli, 1982). In addition, as shown by the data presented in this paper, the CK rate strongly depends on the type of exogenous carbon source. In isovolumic hearts perfused with pyruvate or glucose plus insulin under conditions I and II, the CK flux was ~17 μmol s^{-1} (g dry weight)$^{-1}$. On the other hand, in hearts perfused with glucose (no insulin) at 38 °C, operating at lower RPP (30 000 mmHg/min) and MVO_2, the CK flux was ~27 μmol s^{-1} (g dry weight)$^{-1}$. Clearly, at comparable mechanical performance, the CK flux is more than twice as high in the Langendorff hearts perfused with glucose in the absence of insulin relative to the hearts perfused with pyruvate or with glucose in the presence of insulin. Therefore, in the glucose-perfused hearts, the influence of the ATP $\rightleftarrows$ P_i exchange should be less significant in the CST measurements of CK kinetics. This is probably the reason why the CK flux discrepancy observed by CST is very small or nondetectable (Degani et al., 1985; Bittle & Ingwall, 1985), except possibly under very high work loads (Matthews et al., 1982), in hearts perfused with exogenous glucose and so prominent in hearts perfused with pyruvate, where the CK flux is inherently lower

[6] That is, spin–lattice relaxation time within the intramitochondrial environment but in the absence of any chemical exchange.

even when MVO_2 is very high.

In summary, the data presented in this paper demonstrate that the exchange between ATP_γ and P_i or to be more rigorous, between ATP_γ and a chemical species with the ^{31}P chemical shift of P_i, is the cause of the discrepancy noted in the CST measurements of myocardial CK flux. The simplest operative model which is partially consistent with these data is a three-site exchange of the type $A \rightleftarrows B \rightleftarrows C$.

ACKNOWLEDGMENTS

We are grateful to Robert Thrift for expert help on instrumentation.

Registry No. CK, 9001-15-4; ATP, 56-65-5; CP, 67-07-2; P_i, 14265-44-2; D-glucose, 50-99-7; pyruvate, 127-17-3; insulin, 9004-10-8.

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