Kinetics of the Creatine Kinase Reaction in Neonatal Rabbit Heart: An Empirical Analysis of the Rate Equation†

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ABSTRACT: Here we define the kinetics of the creatine kinase (CK) reaction in an intact mammalian heart containing the full range of CK isoenzymes. Previously derived kinetic constants [Schimerlik, M. I., & Cleland, W. W. (1973) *J. Biol. Chem. 248*, 8418–8423] were refit for the reaction occurring at 37 °C. Steady-state metabolite concentrations from ^{31}P NMR and standard biochemical techniques were determined. ^{31}P magnetization transfer data were obtained to determine unidirectional creatine kinase fluxes in hearts with differing total creatine contents and differing mitochondrial CK activities during KCl arrest and isovolumic work for both the forward reaction (MgATP synthesis) and reverse reaction (phosphocreatine synthesis). The NMR kinetic data and substrate concentration data were used in conjunction with a kinetic model based on MM-CK in solution to determine the applicability of the solution-based kinetic models to the CK kinetics of the intact heart. Our results indicated that no single set of rate equation constants could describe both the KCl-arrested and working hearts. We used our experimental data to constrain the solution-derived kinetic model and derived a second set of rate equation constants, which describe the isovolumic work state. Analysis of our results indicates that the CK reaction is rate limited in the direction of ATP synthesis, the size of the guanidino substrate pool drives the measured CK flux in the intact heart, and during isovolumic work the CK reaction operates under saturating conditions; that is, the substrate concentrations are at least 2-fold greater than the K_m or K_{im} for each substrate. However, during KCl arrest the reaction does not operate under saturating conditions and the CK reaction velocity is strongly influenced by the guanidino substrate pool size.

The adenosine triphosphate creatine phosphotransferase (E.C. 2.7.3.2) (creatine kinase, CK[1]) reaction has been extensively studied in solution. Early studies established the reaction pathways for the purine and guanidino substrates. The work of Morrison and James (1965) and Morrison and Cleland (1966) suggest that the reaction mechanism is rapid equilibrium random bi-bi, in the nomenclature of Cleland (1967). Their investigations were carried out for the MM isoenzyme in solution at pH 8.0 using isotope exchange techniques.

Schimerlik and Cleland (1973) later restudied the reaction at pH 7.0, a pH close to the pH of the cytosol of living cells reported by nuclear magnetic resonance (NMR) spectroscopy (Ingwall, 1982). They found that the double-reciprocal plots relating substrate concentration and reaction velocity exhibited different characteristics at pH 7.0 and pH 8.0 and that the pattern of dead-end complex formation also differed. The kinetic pathway at pH 7.0 was described as rapid equilibrium random forward ordered reverse. These authors postulated that the change in pH altered the binding sites of the enzyme so that at pH 7.0 MgATP must bind before creatine binds. The binding affinities of phosphocreatine (PCr) and MgATP differed, but the binding characteristics of MgADP remained unchanged. Additionally, the enzyme–PCr–MgATP dead-end complex was much more likely to form at pH 7 that at pH 8.

Additional details of the creatine kinase reaction have been delineated by Cook et al. (1981) and Pecoraro et al. (1984). Cook et al. (1981) found that PCr is sticky in the ternary complex (E–MgADP–PCr) and that creatine forms 4–6 times faster than PCr dissociates. However, PCr dissociates 70–80 times faster from the binary complex (E–PCr) than the rate at which product is formed. MgADP is not sticky in the ternary complex (Pecoraro et al., 1984); therefore, its rate of dissociation from the ternary complex is faster than the rate of product formation and release. Taken together these studies invalidate the use of the rapid equilibrium assumption for calculation of rate constants if the pathway E–MgADP → E–MgADP–PCr is active.

The creatine kinase reaction has also been studied in intact tissue by using NMR techniques. Saturation transfer (Bittl & Ingwall, 1985; Perry et al., 1988; Kuprianov et al., 1984; Brindle et al., 1984) and inversion recovery (Meyer et al., 1982; Degani et al., 1985) techniques have been used to determine the velocity of the CK reaction in isolated heart. Brindle et al. (1984) demonstrated excellent agreement between measurements of CK reaction velocity in the direction of ATP

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[1] Abbreviations: BD, baseline deconvolution; CK, creatine kinase; mito-CK, mitochondrial creatine kinase; DW, dry weight; FID, free induction decay; MEFSD, maximum entropy Fourier spectral deconvolution; MEM, maximum entropy method; NMR, nuclear magnetic resonance; PCr, phosphocreatine; T_1, longitudinal relaxation time; WW, wet weight.

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synthesis made by using ^{31}P saturation transfer techniques and ^{15}N isotope-exchange techniques for CK in solution. Degani et al. (1985) demonstrated that saturation transfer and inversion recovery techniques yielded similar values for the forward CK flux both in vitro and in beating Langendorff perfused rat hearts functioning at low workloads, using the T_1 values for PCr and ATP obtained from the inversion recovery sequence. In addition, these authors found the forward and reverse CK fluxes to be the same by both techniques. Bittl and Ingwall (1985), studying beating rat hearts functioning at both high and low workloads, observed workload-dependent differences between forward and reverse CK reaction velocities. These apparent discrepancies can be explained by contributions to ATP synthesis from other reactions (Ugurbil et al., 1986; Spencer et al., 1988).

Measurements of the CK flux in isolated perfused hearts of different species from ^{31}P magnetization transfer indicate that the CK reaction in the heart behaves differently depending on the diversity of CK isoenzymes present. Perry et al. (1988a) found that the forward CK flux measured by NMR during KCl arrest in chicken and ferret heart was the same as that measured during isovolumic work. In contrast, the measured reaction rate was different between the two work states for rabbit and rat heart. All of the data for the chicken, ferret, and rat hearts were obtained from hearts containing significant mitochondrial CK activity. Some of the rabbit data include hearts with no measurable mito-CK activity, but over a limited range of creatine contents.

The ability to measure CK reaction velocity in vivo by using magnetization transfer techniques permits analysis of the accuracy with which the kinetic schemes developed for MM-CK in solution describes the in vivo situation, in which CK activity, isoenzyme distributions, and substrate concentrations vary. To date, only two types of analyses attempting to reconcile measured and predicted reaction velocities have been made. In one, a simple mathematical model allowing for compartmentation of enzyme was developed (Zahler et al., 1987). In the other, the rate equation developed by Morrison and Cleland (1966) was used to compare measured and predicted reaction velocities in in vivo rat brain, heart, and skeletal muscle (Bittl et al., 1987), in isolated rat hearts (Bittl & Ingwall, 1985; Kuprianov et al., 1984), in isolated rabbit heart (Perry et al., 1988), and in isolated ferret heart (Neubauer et al., 1988). Taken together, these studies show that the NMR magnetization transfer measured reaction velocities and the reaction velocities predicted on the basis of solution kinetics do not always agree. Accordingly, in the analysis presented here we have used magnetization transfer measured forward and reverse CK reaction velocities from isolated rabbit hearts with similar total CK activity and isoenzyme distributions but different guanidino substrate contents to ascertain the applicability of the solution-based kinetic schemes to the intact heart during KCl arrest and isovolumic work. The form of the rate equation is the rapid equilibrium model of Schimerlik and Cleland (1973), modified to account for the work of the Cook et al. (1981). The NMR-derived reaction velocity data indicate that two different sets of rate equation constants need to be specified in order to describe the CK reaction kinetics in both KCl-arrested and working hearts. The NMR-measured forward and reverse reaction velocities were used as constraints to calculate a set of rate equation constants that describe the CK kinetics in the working heart. In this way, we used experimentally derived reaction velocities, enzyme activity measurements, and substrate concentrations to determine a set of kinetic constants that provide agreement for the predicted values of V_{max} for the two work states. Finally, we used the rate equation to analyze the dependency of V_{max} on guanidino substrate and other factors and to speculate on the impact of the change in kinetic constants on CK regulation in vivo.

To obtain the necessary experimental data we studied isolated hearts from rabbits 3–5 days old. During this time there is a change in CK isoenzyme distribution and total guanidino substrate content but not total CK activity. NMR measurements of forward and reverse reaction velocities and steady-state substrate concentrations were obtained during KCl arrest and isovolumic work. These data were combined with standard biochemical assays of total creatine content and CK activity to yield the analysis herein. A preliminary report of a portion of this work has been previously presented (McAuliffe et al., 1989).

MATERIALS AND METHODS

New Zealand white rabbits age 3–5 days were used according to protocols approved by the IACUC at each institution. The animals were anesthetized with intraperitoneal pentobarbital (50 mg/kg). The thorax was entered and heparin (100U) supplied via the right atrium. The ~300-mg heart was rapidly excised and perfused retrograde via the aorta. A pulmonary arteriotomy incision was made, and a vent and an isovolumic balloon were placed in the left ventricle. The last step was omitted in those hearts that were studied under conditions of KCl arrest. The perfusate used was a phosphate-free Krebs–Henseleit buffer equilibrated with 95% O_2/5% CO_2 at pH 7.4 at 37 °C as previously described (Bittl & Ingwall, 1985; Perry et al., 1988). Two conditions were studied: hearts arrested with KCl and hearts doing isovolumic work at a rate–pressure product (the product of heart rate and left ventricular developed pressure) of ~10 000 torr min^{-1}. KCl arrest was achieved by increasing the concentration of K^+ in the buffer to 20 mmol L^{-1}. Intermediate workloads were not studied as the major change in measured CK reaction flux occurs with the transition from KCl arrest to isovolumic work (Bittl & Ingwall, 1985; Perry et al., 1988b). The value of 10 000 torr min^{-1} for the rate–pressure product represents approximately 60–75% of the maximum sustainable workload for isolated isovolumic neonatal rabbit hearts.

Hearts were placed in fixed-frequency NMR probes tuned to ^{31}P and physiologic parameters were recorded simultaneously by using a Statham transducer interfaced to either a Hewlett-Packard strip chart recorder or to a Grass Physiograph recorder. ^{31}P NMR spectra were obtained by using either a Nicolet NT 360 89-mm bore spectrometer or a NT 300 31-mm bore spectrometer, each interfaced to a Nicolet 1280 computer. The acquisition parameters for the NMR experiments were a flip angle of 72° for 256 acquisitions, using quadrature phase detection. The saturating pulse used for the $M_{infinity}$ block was 5 s long. The pulsed Fourier transform mode was used in all NMR experiments.

Saturation Transfer Experiments. The forward CK flux (in the direction of ATP synthesis) was measured by saturating the [γ-P]ATP resonance and the reverse flux (in the direction of PCr synthesis) was measured by saturating the PCr resonance. Two types of saturation transfer experiments were used. These have been previously described (Perry et al., 1988) and referred to as SELSAT and MOMZ. Briefly, the SELSAT experiments employed progressively longer saturating pulses and yield data that allow estimation of the parameters M_0, $M_{infinity}$, and τ, the apparent NMR lifetime of either PCr or ATP. The values of the spin–lattice relaxation times, T_1, and the pseudo-first-order rate constants were derived as in Bittl

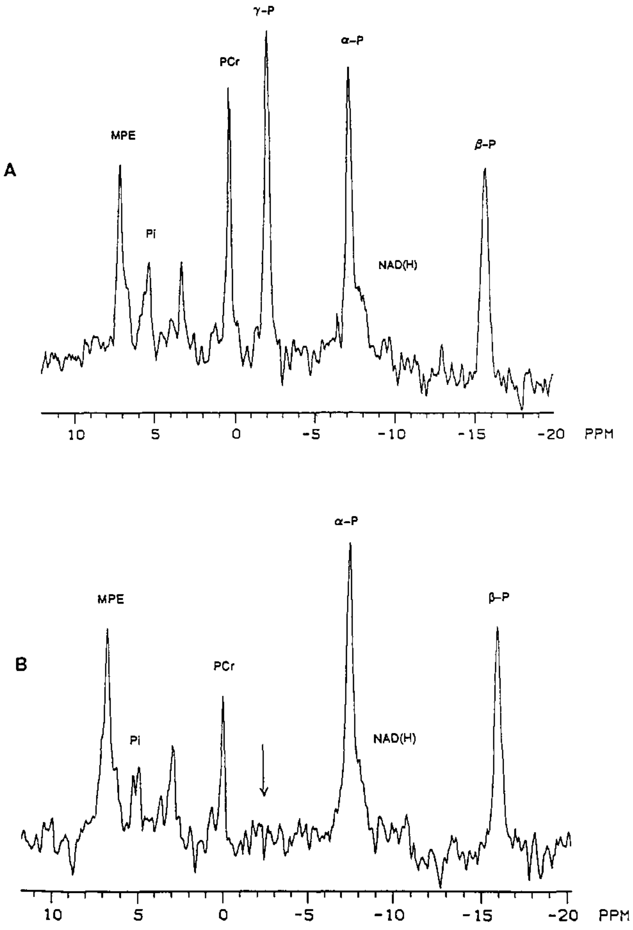

FIGURE 1: MOMZ-type experiment performed using the NT-300 system employing an external synthesizer as the source of the saturating signal. The [γ-P]ATP resonance is saturated (arrow), which results in a decrease in the intensity of the PCr resonance. The magnitude of the decrease is related to the rate of chemical exchange between the two species.

and Ingwall (1985) using a two-site exchange model. The MOMZ experiments utilized alternate block acquisitions of a pulse sequence without a saturating pulse and one with an "infinitely long" selective saturating pulse (5 s). In this sequence, the interval between hard pulses is constant. The experiments performed on the NT 300 system employed an external frequency synthesizer (PTS 250) that used the same 10-MHz standard as the spectrometer. The pulse timing and duration were controlled by the pulse programmer using TTL gating. The experiments performed on the 360-MHz system utilized the lower power broad-band unit contained in the spectrometer.

Prior to the initiation of the saturation transfer studies, the selectivity of the low power saturating pulses was tested to assess the degree of saturation of adjacent resonances. At a frequency offset of 200 Hz there is less than 5% saturation of adjacent resonances; while there is better than 97% saturation at zero offset. The frequency separation between the PCr and the [γ-P]ATP resonances is 300 Hz for the NT 300 system. Thus the saturating signal will have no effect on either of these resonances when the other is saturated except as mediated by chemical exchange. Spectra from a representative MOMZ experiment obtained on the NT 300 where the [γ-P]ATP resonance was saturated are shown in Figure 1.

SELSAT experiments were performed under conditions of KCl arrest and the data were used to derive the intrinsic longitudinal relaxation time, T_1, for PCr. Since the T_1 value for PCr does not vary with work (Bittl & Ingwall, 1985; Perry et al., 1988b), these T_1 values were used with the data obtained from the MOMZ experiments to calculate the forward CK reaction velocity (i.e., in the direction of ATP synthesis) for both KCl-arrested and working hearts. SELSAT experiments were used for both KCl arrested and working hearts to obtain values for the reverse reaction velocity (in the direction of PCr synthesis). Measurements for forward and reverse flux from the same heart under conditions of isovolumic work were also obtained by using the MOMZ protocol. The results of the MOMZ and SELSAT experiments were pooled, as these two protocols have been previously shown to give equivalent results (Perry et al., 1988b).

Biochemical Analysis. Hearts were frozen in liquid nitrogen at the conclusion of each experiment and stored at −70 °C. They were subsequently analyzed for total protein content, total CK activity and isoenzyme distribution, and total creatine and ATP contents by using previously described techniques (Perry et al., 1988b). Nonperfused hearts from rabbits of the same age as those used in the NMR experiments were also subjected to the same analysis to ensure that the perfusion conditions did not result in any significant loss of enzyme activity or substrates.

Data Processing. Taken together, data from the NMR experiments and the biochemical analysis provide measurement of the steady-state metabolite concentrations of MgATP, PCr, creatine, and the forward and reverse CK fluxes. The MgADP concentration was calculated by assuming CK equilibrium and using measured values for ATP, PCr, and creatine concentration, the pH determined for each heart from the chemical shift of the P_i peak relative to the PCr resonance, and the equilibrium constant of Veech et al. (1979) as previously described. The steady-state contents of ATP and PCr were used as the M_0 values to calculate forward and reverse fluxes through the CK reaction.

All NMR spectra were acquired in quadrature and initially processed by using the following sequence: baseline correction, exponential multiplication, and Fourier transformation followed by manual phasing. A line broadening of 20 Hz was used. The standard Nicolet integration package or its equivalent was used to derive quantitative values for resonance areas used to calculate metabolite concentrations and rate constants. This approach proved to be unsatisfactory for some data sets. Specifically, spectra acquired on the NT 300 for working hearts were difficult to integrate accurately because of low signal to noise ratios for some resonances. This was especially true for the measurement of the forward flux since the PCr resonance area, characteristically small in the neonatal rabbit heart, is further reduced during saturation transfer experiments. This was not a problem for the KCl-arrested hearts or for the reverse flux experiments. Accordingly, the data sets for the foward flux experiments using the MOMZ sequence for the working hearts were subjected to a data processing routine using the software modules NMR1 and MEM/LPM of New Methods Research, Inc. (Syracuse, NY). The FID's were initially processed as above. Subsequent processing included baseline deconvolution (BD) and maximum entropy Fourier spectral deconvolution (MEFSD). Integration was performed by using Simpson's rule to avoid error introduced by fitting the non-Lorentzian lines with an integration routine that is based on Lorentzian line shapes. The result of this processing routine was a dramatic improvement in signal to noise ratio, improved spectral resolution, and eliminated the baseline ambiguity that is present in conventionally processed spectra (Sibisi et al., 1984). Representative comparisons of conventionally processed and BD-MEFSD

Table I: CK Activity Profile of Perfused Hearts[a]

group	CK (IU/mg of protein)	isoenzymes			creatine (μmol/g)
		BB + MB	MM	mito	
1. KCl arrest (n = 5)	11.0 ± 2.1	25.5 ± 5.9	74.5 ± 4.9	0.0 ± 0.0	22.2 ± 3.3
ANOVA	NS	4	NS	4	2
2. KCl arrest (n = 6)	9.8 ± 1.3	17.5 ± 7.1	82.5 ± 7.3	0.0 ± 0.0	38.1 ± 10.2
ANOVA	NS	NS	NS	4	1, 3, 4
3. working (n = 5)	9.2 ± 1.3	24.9 ± 5.2	75.1 ± 5.2	0.0 ± 0.0	18.3 ± 2.5
ANOVA	NS	4	NS	4	2
4. working (n = 5)	9.6 ± 1.5	15.2 ± 3.2	82.6 ± 7.3	1.9 ± 0.2	22.1 ± 2.4
ANOVA	NS	1, 3	NS	1, 2, 3	2

[a] Mean values ± SD. ANOVA results: NS, no significant difference between that group and any other. The entries indicate the group in the column that is different at the 95% confidence level. For example, the 4 in the CS column for group 3 indicates that group 4 is different from group 3 at the 95% confidence level.

processed spectra are shown in Figure 2. Studies of phantom systems have shown no difference in the relative peak areas for spectra processed either conventionally or by using BD followed by MEFSD.

Statistical Analysis. Comparisons between two groups were made by using a two-tailed "t" test. A p value of <0.05 was taken as significant. Multiple comparisons were done by using analysis of variance (ANOVA). The estimates of $V_{\max}$ obtained by substitutions of metabolite concentrations and dissociation constants into the rate equations were grouped as described and the data for groups were compared by using ANOVA (SAS Institute Inc, Cary, NC, release 5.16). The appropriate parametric or nonparametric test was applied as indicated by the results of the Wilk-Shapiro W test. The Scheffe test was used to determine the significance of the differences in mean values for each group as the groups were of different sizes when parametric analysis was appropriate. The corresponding nonparametric test was the method of Dunn (Hollander & Wolfe, 1973), which defines a critical value for the difference between the Wilcoxon rank sum mean scores given in the SAS ANOVA output. This test was applied only if the Kruskal-Wallis χ-squared test indicated significance, otherwise there was no significant difference between any pair of the groups entered in the ANOVA data table. Data were presented as means ± one standard deviation.

RESULTS

Optimization of Perfusion Conditions. The perfusion conditions for isolated neonatal hearts were optimized by adjusting the rate of perfusate flow to yield the maximum value for the PCr resonance area. Flow rates of 14–19 mL min^{-1} $g_{ww}{}^{-1}$ provided maximum values for the PCr resonance area and for the ratios of the resonance areas of PCr to P_i and PCr to [β-P]ATP. The results of the perfusion experiments relating PCr resonance area and flow are shown in Figure 3. The stability of the preparation was tested by obtaining serial spectra over the course of 3 h of isovolumic work. The PCr and ATP resonance areas were stable for at least 3 h at rate pressure products of 10 000 torr min^{-1}.

Biochemical Characteristics of Neonatal Hearts. In this study, 11 arrested and 10 working hearts were analyzed; their enzymatic profiles defining the CK system are shown in Table I. All hearts had similar values for total CK activity. Consistent with maturation of the CK system during early neonatal development in rabbit heart, the CK isoenzyme distribution and total creatine content differed among hearts, forming the basis for dividing the hearts among four groups. Six of the 11 KCl-arrested hearts had a relatively high total creatine content. Five of the 10 working hearts were the only hearts to contain mitochondrial CK activity (~2% of the total CK activity). Thus, based on differences in workload, CK isoenzyme distribution, and total creatine content, four groups were defined: arrested hearts with either low (group 1) or high (group 2) creatine content and working hearts with either no (group 3) or barely measurable (group 4) mitochondrial CK activity. Groups 1, 2, and 3 had similar isoenzyme distributions and, compared to adult mammalian myocardium, all groups had relatively high MB isoenzyme, thus forming the

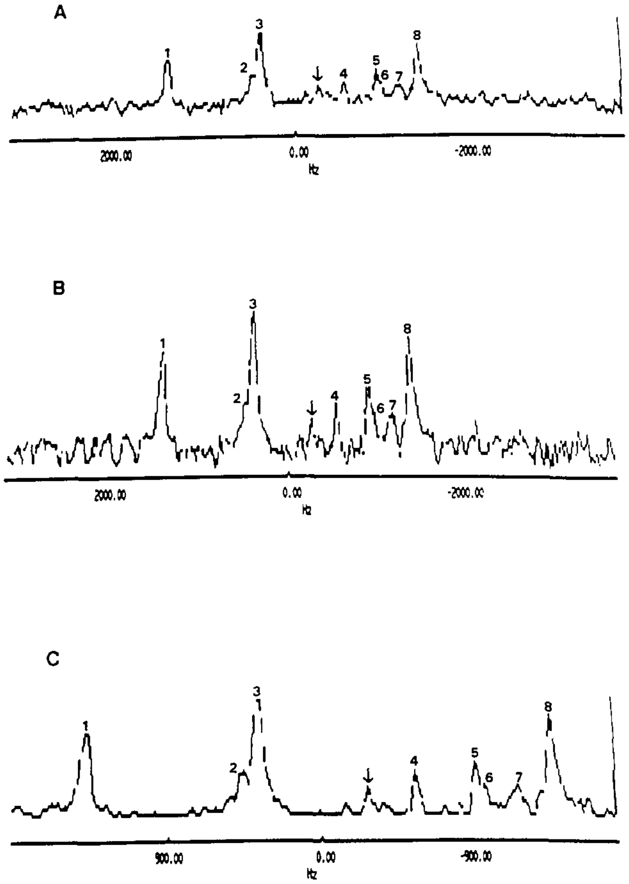

FIGURE 2: Side by side comparison of a Fourier transform (FT) processed spectra of a working heart, panel A, and the same free induction decay (FID) processed by using MEFSD (see text for details), panel C. The baseline → deconvolved spectrum, the intermediate step, is shown in panel B. The spectrum shown is a M_{infinity} block of a MOMZ experiment using a working heart with 0% mito-CK. The peaks are (1) [β-P]ATP, (2) NADP(H), (3) [α-P]-αATP, (4) PCr, (5) glycerophosphocholine, (6) glycerol phosphoethanolamine, (7) inorganic phosphate, and (8) phosphomonoesters. The [γ-P]ATP peak, ↓, is not completely saturated; however, the residual areas is only about 5–10% of the original intensity. The PCr peak is easily identified and has a well-defined baseline in the MEFSD spectrum. Errors here are likely to overestimate peak area and therefore reduce the calculated CK flux, thus minimizing the difference between the working and KCl-arrested hearts.

Table II: Metabolite Concentrations (mM) for CK Reaction[a,b]

group	pH	ATP	Creatine	PCr	ADP
1. KCl arrest	7.22 ± 0.02	8.1 ± 0.7	5.9 ± 1.2	2.7 ± 0.9	0.16 ± 0.09
ANOVA	3, 4	NS	4	2	NS
2. KCl arrest	7.23 ± 0.02	7.9 ± 0.4	8.2 ± 3.1	5.9 ± 0.8	0.11 ± 0.04
ANOVA	3, 4	NS	3, 4	1, 3, 4	NS
3. working (0% mito)	7.10 ± 0.02	7.1 ± 1.0	4.5 ± 0.5	2.2 ± 0.3	0.11 ± 0.01
ANOVA	1, 2, 4	NS	2	2, 4	NS
4. working (1.9% mito)	7.16 ± 0.01	7.8 ± 0.5	4.2 ± 0.2	3.3 ± 0.2	0.08 ± 0.01
ANOVA	1, 2, 3	NS	1, 2	2, 3	NS

[a] Mean value ± SD. These calculations assume a wet weight to dry weight ratio of 5.4 and an intracellular water content of 1 g of water per 2 g wet wt. [b] ANOVA results: The numbers in each cell indicate the group or groups in each column that are different at the 95% level; e.g., under pH for group 1 the numbers 3, 4 appear indicating that the pH of group 1 is significantly different from those of groups 3 and 4. NS means there is no group in the column that is different from any other at the 95% confidence level.

Table III: Summary of CK Flux Measurements[a,b]

group	[PCr] (μmol/g_{DW})	K_f (s^{-1})	$flux_f$ [μmol/(g_{DW} s^{-1})]	[ATP] (μmol/g_{DW})	K_r (s^{-1})	$flux_r$ [μmol/(g_{DW} s^{-1})]
1. KCl arrest (n = 5, 4)	7.3 ± 2.4	0.67 ± 0.22	4.4 ± 0.6	21.1 ± 1.5	0.22 ± 0.04	4.6 ± 1.0
ANOVA			2, 3, 4			
2. KCl arrest (n = 6, 3)	16.4 ± 2.3	0.61 ± 0.09	9.8 ± 1.8	22.0 ± 1.2	0.42 ± 0.06	9.2 ± 0.6
ANOVA			1, 4			
3. working (n = 5, 4)	6.0 ± 0.7	1.57 ± 0.27	9.3 ± 0.6	20.2 ± 1.6	0.44 ± 0.09	8.8 ± 0.9
ANOVA			1, 4			
4. working (n = 4, 5)	9.3 ± 1.1	1.36 ± 0.13	12.6 ± 0.8	21.4 ± 1.2	0.56 ± 0.04	12.0 ± 0.33
ANOVA			1, 2, 3			

[a] Mean values ± SD. The numbers in parentheses refer to the number of hearts used for the forward and reverse flux measurements in each group. The forward and reverse flux values are identical within experimental error for each group. [b] The ANOVA results are presented as in the previous tables.

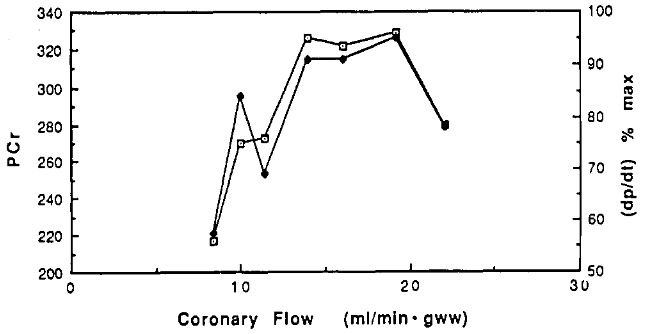

FIGURE 3: Relationship between perfusate flow rate and the intensity of the PCr resonance (open squares) and the contractility expressed as dp/dt (closed diamonds) is shown. Each data point is the average of at least two measurements from each of three hearts perfused over a 2-h period. The scale for the PCr resonance is in arbitrary units with the area of the [β-P]ATP resonance defined as 1000 units. The ATP content of the neonatal rabbit heart is 22 μmol g_{DW}^{-1} on average. The scale for dp/dt is normalized against the maximum value recorded for any heart studied during the optimization protocol. All hearts used in the remainder of the study were perfused at a rate of 16–20 mL/(min g_{WW}).

basis for analysis of the CK reaction for the two work states that follows.

Metabolite concentrations for the CK reaction, expressed as cytosolic concentration, for each of the four groups are shown in Table II. ATP concentration was the same for all groups, but values for the [PCr] and the PCr/free creatine ratio (and hence ATP/ADP ratio) differed. Hearts with the highest total creatine content contained the most PCr (group 2). Group 4 hearts with the most adultlike CK isoenzyme distribution had the highest PCr/free creatine ratio and consequently the highest ATP/ADP ratio. Values for pH of both groups of KCl-arrested hearts were slightly more alkaline than for working hearts.

Magnetization Transfer Results. The results of the SEL-SAT experiments yielded the data necessary to calculate values for the T_1 of PCr and [γ-P]ATP as well as the forward and reverse CK reaction rate constants and unidirectional fluxes. The intrinsic longitudinal relaxation time, T_1, of PCr was 2.1 ± 0.3 s. The T_1 of ATP did not change significantly with change in cardiac workload and averaged 0.9 ± 0.2 s.

Mean values for the forward and reverse CK pseudo-first-order rate constants and unidirectional fluxes, as well as the ratio of the forward to reverse fluxes, for each group of hearts are shown in Table III. Since the forward and reverse rate constants, K_f and K_r, were measured in the same heart whenever possible, paired comparisons were made by using data from each heart. For each group of hearts, regardless of workload, creatine content, or CK isoenzyme distribution, the forward CK flux was indistinguishable from the reverse flux. However, the absolute value of CK flux differed among groups. Several comparisons can be made. For the same creatine content and isoenzyme distribution (compare group 3 to 1), CK flux is greater in working than in arrested hearts. But, at the same workload, flux is greater in hearts with higher creatine content (compare group 2 to 1) and with more mature CK isoenzyme distribution (compare group 4 to 3). Equality of CK flux in arrested hearts containing high guanidino levels with CK flux in working hearts with a lower guanidino content (a finding not previously described) emphasizes the role of the guanidino pool in driving the CK reaction. The pseudo-first-order rate constant for the forward reaction, K_f, was related to cardiac work and was not related to guanidino substrate concentration or the ratios of the guanidino or purine pools (compare groups 1 and 2 versus 3 or 4). The reverse flux constant K_r is a guanidino substrate dependent constant by mathematical necessity. However, at constant guanidino substrate concentration, it is also work dependent (compare groups 1 and 3).

The actual rate constants for the forward CK reaction were derived by dividing the pseudo-first-order constant by the concentration of the implicit reactants, i.e., MgADP and H^+. The actual rate constant for the KCl-arrested hearts is 7.2×10^{10} M^{-2} s^{-1} and is 22×10^{10} M^{-2} s^{-1} for the working hearts. The difference between these values is significant. This indicates that the differences between the pseudo-first-order ratio

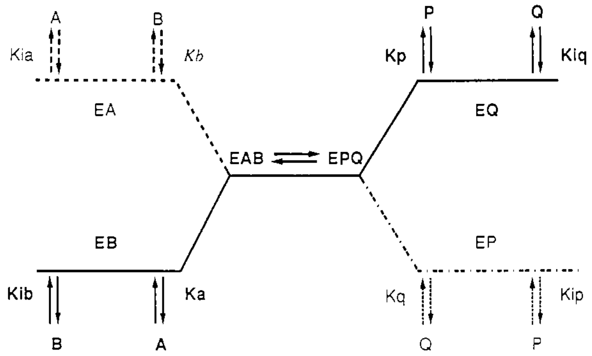

FIGURE 4: Proposed reaction scheme for the creatine kinase reaction in the intact heart is shown. The in vitro reaction scheme is shown at pH 7 (- - -) and at pH 8 (- · -) lines. The in vivo scheme is in solid lines; the constants used for the in vivo scheme are in bold type. The constant K_b is in italics because it is not a true constant, as the rapid equilibrium assumption is not valid for this step. The convention of Cleland (1963) is used for the symbols *A*, *B*, *P*, and *Q*. The in vivo reaction scheme is based on substrate concentration data and constants derived from the in vitro data of Morrison and Cleland (1966) and Schimerlik and Cleland (1973). These constants are given in Table VI of the Appendix. This analysis predicts that the reaction is ordered in both directions and is referred to as the rapid equilibrium ordered (REO) scheme.

constants is the result of an actual change in the kinetics and not merely the result of change in implicit reactant concentrations.

Derivation of Rate Equations for the CK Reaction under Physiological Conditions. The form of the CK reaction scheme used to describe the reaction in the intact heart is the same as that proposed by Schimerlik and Cleland (1973) for MM-CK in solution pH 7, as this is near the pH in the cytosol of the heart, modified to account for the work of Cook et al. (1981), which indicates that the K_m of PCr is functionally zero. This eliminates the pathway E → E–MgADP → E–MgADP–PCr from the pathway given by Schimerlik and Cleland (1973). Those authors showed that the pathway E → E–Cr → E–Cr–MgATP is not functional at physiological pH. These considerations reduce the kinetic scheme to a rapid equilibrium ordered reaction in both directions with the guanidino moiety adding first in the direction of MgATP synthesis and the nucleotide adding first in the direction of PCr synthesis (Figure 4). It is important to note that the exact form of the rate equation used does *not* alter the conclusions drawn herein concerning the kinetics of the CK reaction in the neonatal rabbit heart. We have analyzed the data using the full general form of the rapid equilibrium random scheme of Morrison and Cleland (1966) and obtained the same results as shown in Table IV (not shown).

The values of the dissociation constants to be used in the rate equations were calculated by adjusting the data of Morrison and James (1965) and Schimerlik and Cleland (1973) to account for the increase in reaction rate expected for a temperature change from either 30 or 25 °C used in their solution experiments to 37 °C used for the in vivo experiments described here. A Q_{10} of 2.5 (empirically derived) was used to estimate the increase in CK reaction velocity with temperature. The recomputed reaction rates and experimental substrate concentrations were then fit to the appropriate initial velocity equations [Schimerlik and Cleland (1973), eqs 1 and 2] by using a nonlinear least-squares regression (BMDP Statistical Software, Inc., University of California, Los Angeles, revised 1987). This procedure yielded values for all Michaelis constants and dissociation constants at 37 °C for both pH 8 and pH 7. The values for the inhibition constants were used with little or no change from the originally published values. The values for the dissociation constants at 37 °C used in these calculations are presented in Table VI in the Appendix.

Table IV: Forward V_{max} Values Calculated from the Rate Equation Analysis[a]

group	case 1 forward	case 2 forward	case 2 reverse
1. KCl arrest (0% mito-CK)	28 ± 6	5.3 ± 1.7 (5.3)	21.1 ± 4.6
ANOVA	2, 3, 4	2, 3, 4	
2. KCl arrest (0% mito-CK)	54 ± 12	12.3 ± 3.2 (5.5)	35.0 ± 8.6
ANOVA	1, 3, 4	1, 4	
3. working (0% mito-CK)	92 ± 15	15.1 ± 1.5 (17.2)	31.3 ± 3.4
ANOVA	1, 2, 4	1, 4	
4. working (1.9% mito-CK)	120 ± 8	24.4 ± 2.1 (18.5)	58.2 ± 4.7
ANOVA	1, 2, 3	1, 2, 3	

[a] The tabulated values are the mean and standard deviations for the estimated V_{max} generated by substituting substrate concentrations and CK flux measurements into the rate equations by using the constants in the Appendix. Case 1 is rapid equilibrium ordered at pH 8; case 2 is REO at pH 7. The numbers in parentheses indicate the V_{max} if it is normalized for [PCr]. The ANOVA results are presented as in the previous tables.

To confirm that the mathematical manipulation was correct, we fit the temperature-adjusted data reported by Morrison and James (1965) for MM-CK in solution at pH 8.0 to the initial velocity equations and calculated V_{max} in the direction of PCr + MgADP to products. We obtained a value of 0.346 mM s^{-1}, which corresponds to a V_{max} of 0.197 mM s^{-1} at 30 °C. This is similar to the value of 0.208 mM s^{-1} given for MM-CK in solution in the original publication. The estimated V_{max} for the CK reaction in the opposite direction was 0.196 mM s^{-1} at 37 °C. This corresponds to a value of 0.112 at 30 °C and agrees very well with the value of 0.111 obtained by Morrison and James (1965). The corresponding values obtained by refitting the temperature-adjusted data of Schimerlik and Cleland (1973) are V_{max} = 0.89 mM s^{-1} for MgATP synthesis and V_{max} = 1.76 mM s^{-1} for PCr synthesis.

These calculations for MM-CK in solution showed that the V_{max} is greater in the direction of ATP synthesis at pH 8, while at pH 7 the V_{max} is greater in the direction of PCr synthesis. Thus at pH 7 the phosphorylation of MgADP to MgATP would appear to be limiting. This corresponds to the forward flux direction in the present heart study. Since all hearts studied had intracellular pH values of about 7, the phosphorylation of MgADP to MgATP should be the limiting step in heart as well as in solution. This is what was observed (Table IV).

Application of the Rate Equation to the CK Reaction in Vivo. The rapid equilibrium ordered form of the rate equation was used in conjunction with the temperature-adjusted constants (Table VI, Appendix) and the metabolite concentrations from Table II to determine the predicted forward V_{max} for two cases for each group of hearts studied, case 1 using the pH 8 constants and case 2 using pH 7 constants. The summary of the rate equation calculations given in Table IV lists the V_{max} values for groups 1–4 for both the pH 7 and 8 constants. For the pH 7 case, the V_{max} for both the forward and reverse reactions is listed. The V_{max} for the reverse reaction is greater than that of V_{max} forward, indicating that the CK reaction in the intact heart is rate limited in the direction of ATP synthesis.

To compare data obtained for group 2 hearts, which contain higher amounts of total guanidino substrate, and the other groups, we determined the dependence of V_{max} on [PCr]. The dependence of V_{max} on [PCr] can be determined by solving for the partial derivative of V_{max} with respect to [PCr] (not shown). The value of V_{max} is linearly dependent upon [PCr]. On the basis of cytosolic substrate concentration, the V_{max} expected for group 2 can be determined by multiplying the

Table V: Predicted V_{max} Values from Revised Constants[a]

group	% mito-CK	V_{max} (forward) ($mM\ s^{-1}$)	V_{max} (reverse) ($mM\ s^{-1}$)
1. KCl arrest	0	5.3 ± 1.5 (5.1)	21.1 ± 4.6 (21.1)
2. KCl arrest	0	12.3 ± 2.4 (5.6)	35.0 ± 8.6 (16.0)
3. working	0	5.2 ± 0.6 (5.2)	20.4 ± 2.87 (20.4)
4. working	1.9	6.3 ± 0.5	25.0 ± 1.6

[a] The V_{max} values listed were calculated by using the rapid equilibrium ordered kinetic scheme and the pH 7 constants for the cytosolic isoenzymes of CK. The values for K_a, K_b, and K_p for the KCl-arrested heart groups were taken from the Appendix. For the working hearts the constants are $K_a = 0.003$, $K_{ib} = 1.05$, $K_p = 1.46$, and $K_{iq} = 1.0$. These were obtained by solving simultaneous equations constrained as discussed in the text. The numbers in parentheses are the mean V_{max} values calculated for each group if V_{max} is corrected for the effect of PCr. Only the 0% mito-CK hearts have been corrected for [PCr] as one effect of mito-CK is to increase [PCr] at constant total creatine.

V_{max} for group 1 by the ratio $[PCr]_{group2}/[PCr]_{group1}$. By use of this analysis, the V_{max} values for groups 1 and 2 of case 2 became identical (Table IV). However, the V_{max} values for the working hearts are still significantly different from those for the KCl-arrested hearts. Thus, differences in substrate concentrations can account for the differences in V_{max} at a given workload (compare groups 1 and 2) but *cannot* explain the differences in experimentally observed V_{max} with change from arrest to beating conditions. The small difference in pH between the KCl-arrested and working hearts is insufficient to account for the differences in V_{max} observed. The same result is obtained regardless of the kinetic model used if the same set of dissociation and Michaelis constants is used to describe both the KCl-arrested state and the isovolumic work state (calculations not shown).

As is apparent from inspection of Table IV, the V_{max} values predicted for the KCl-arrested hearts are different for those predicted for the working hearts at constant substrate concentration (group 1 versus 3). The unidirectional CK flux measured by NMR should be equiavlent to the initial reaction velocity in solution measured by isotope techniques. The forward flux, v, is the [PCr] times K_f, and v in the rate equation is given by $v = V_{max}(EAB)/E_t$; then the pseudo-first-order rate constant K_f is given by the expression $K_f = V_{max}E(A)/(E_tK_aK_{ib})$, where E is the free enzyme concentration, E_t is the total of all enzyme forms, A is [MgADP], and K_a and K_{ib} are the Michaelis and binary dissociation constants for MgADP and PCr, respectively. The data indicate that [MgADP] is essentially the same for groups 1, 2, and 3. Since isoenzyme distributions are equivalent for groups 1, 2, and 3, all pH values are within a narrow range, and there are no data to indicate that the CK reaction is inhibited in the KCl-arrested hearts, V_{max} should be identical for both the KCl-arrested and working hearts. If V_{max} is the same for both KCl arrest and work, then the E/E_t relationship or K_a and K_{1b} must change. A change in E/E_t implies a change in the rate equation constants as E/E_t is determined by the substrate concentration and the rate equation constants K_m and K_{im} (see the Appendix).

A similar set of equations can be worked out for the reverse reaction. If the NMR-derived reaction velocities and experimentally determined substrate concentrations are used to constrain the set of equations for K_f and K_r and V_{max} is set to that calculated for the KCl-arrested hearts by using the temperature-adjusted solution-derived rate constants, then a new set of rate equation constants describing the isovolumic work state is obtained.

These constants are shown in Table V and have been used to calculate V_{max} for the working hearts by using the measured substrate concentrations and NMR-measured reaction velocities for both the forward and reverse reactions. By use of these constants, the V_{max} values for the KCl-arrested hearts and the working hearts are now equal when the effect of the guanidino substrate pool size is taken into account.

The effect of changes in isoenzyme distribution on V_{max} can be assessed by comparing data for all groups corrected for the guanidino substrate pool size (Table V). The range of isoenzyme distributions in the 3–5-day-old rabbit hearts studied is 15–26% BB + MB, 74–83% MM, and 0–2% mito-CK. Over this range, we find no effect of isoenzyme distribution on V_{max} using the reaction scheme and dissociation constants based on the experimentally derived data. The corrected V_{max} for the group 4 hearts for both the forward and reverse reactions is not significantly different from the V_{max} of the hearts containing no measurable mito-CK. This is in contrast to prior findings in older neonatal hearts, as the mito-CK content of older neonatal rabbit hearts has been previously shown to affect the value of V_{max} (Perry et al., 1988b). Our analysis also suggests that in the newborn rabbit heart all nonmitochondrial CK isoenzymes function similarly.

DISCUSSION

NMR Spectral Analysis. The choice of data processing routines for NMR spectra merits some comment before discussing the data and conclusions. The use of maximum entropy methods (MEM) for processing of low signal to noise data has been found to faithfully reproduce relative peak intensities in studies using simulated data (Sibisi et al., 1984). MEM should reproduce the original data points, the free induction decay, more accurately than apodization and Fourier transform. However, the presence of baseline distortion and phase anomalies may limit its use (Martin, 1985). The scheme chosen here was designed to remove both phase and baseline anomalies before employing the MEM algorithm, in this case, MEFSD. This approach has been found to yield excellent resolution and distortion-free baselines in studies of segments of ^{13}C NMR spectra with low signal to noise ratios (Mazzeo et al., 1989) and we show here that it allows quantitative analysis of ^{31}P NMR spectra with low signal to noise ratios (Figure 3).

Magnetization Transfer Results. An interesting result of the present study is that in the absence, or at low levels, of the mitochondrial CK isoenzyme, the velocities of the forward and reverse CK reaction are equal in both arrested and working hearts. Previous experiments in adult rat hearts have shown that the forward and reverse fluxes are equal only at low workloads (Bittl & Ingwall, 1985). The difference between our results in the newborn rabbit heart and the results in adult rat hearts may relate to maturation-dependent differences in CK isoenzyme distribution and guanidino content. Adult rat heart has high percentages of total CK as the mitochondrial (25%) and MB (12%) isoenzymes and a high guanidino substrate content; newborn rabbit heart also contains high MB (25%) but little or no mito-CK and has a low guanidino substrate content. The CK reaction at the mitochondrial membrane is postulated to operate predominantly in the direction of PCr synthesis (reverse reaction). The equality of forward and reverse flux in the absence of the mito-CK isoenzyme observed here suggests that the NMR-measured flux is largely a reflection of the rate of the CK reaction outside the mitochondrial space. This is also supported by the data of Bittl and Ingwall (1985) and Perry et al. (1988b), which indicates that the CK flux is at least 7–10 times the rate of ATP production by oxidative phosphorylation. The rate of ATP production is the upper limit of PCr pro-

Table VI: Summary of Constants Used in Rate Equations

	cytosolic isoenzymes		
	pH 7.0	pH 8.0	mito-CK
	Michaelis Constants (mM at 37 °C)		
K_a	0.04	0.05	0.05
K_b	0.46 (0)	3.53	2.0
K_p	11.8	6.09	17.3
K_q		0.44	0.93
	Dissociation Constants (mM at 37 °C)		
K_{ia}	0.30	0.17	(0.17)
K_{ib}	4.2	10.7	1.4
K_{ip}	$>10^{10}$	41	29.4
K_{iq}	4.17	1.33	0.29
	Inhibition Constants (mM at 37 °C)		
K_{Ib}	3.9	18	
K_{Ip}	55	24	

duction from ATP produced by oxidative phosphorylation via the mito-CK reaction. In the present study, the rate of flux through other reactions competing with the CK reverse reaction for ATP must be relatively low so that the two exchange site approximation is valid.

Rate Equation Analysis. Our choice of the rapid equilibrium ordered reaction scheme is based on studies of MM-CK in solution and the NMR observation that the pH of the cytosolic space of the heart is near 7. We interpreted the data of Cook et al. (1981) to indicate that at physiologic pH and temperature the CK reaction is not rapid equilibrium random in the direction of MgATP synthesis. While this assumption may be debatable, the fundamental conclusions of this report are not altered.

The data and analysis indicate that the NMR-measured whole-organ enzymology of the CK reaction in the neonatal rabbit heart conforms to a solution-derived reaction scheme only if the numerical values of the binary dissociation and Michaelis constants decrease with increasing cardiac work. Thus, two set of constants are specified to describe both the KCl-arrested state and the isovolumic work state. It is important to note that these are lumped parameter constants; that is, they represent the sum of the contributions of the constants due to individual isoenzymes weighted by the activity of the isoenzyme in question. The data do not permit us to dissect the individual contributions of the isoenzymes present, but MB and MM-CK appear to act similarly in the hearts studied.

The numerical value of the set of constants calculated for working hearts is constrained by the NMR-measured forward and reverse flux measurements and the rate equation itself. The exact value will vary slightly with the form of the rate equation, but no matter which form is used *two* sets of Michaelis and binary dissociation constants need to be specified in order to describe the CK kinetics in the intact heart for both work states. This is demonstrated in Table V as use of two sets of rate equation constants results in a V_{max} that is numerically equal for all groups, after correction for substrate concentration effects, for *both* the forward and reverse reactions. The rate equation constants obtained by refitting the in vitro data to 37 °C have been assumed to describe the KCl-arrested state.

The V_{max} predicted for groups 1 and 3 in Table V (14.5 μmol g^{-1} s^{-1}) is similar to the V_{max} (18 μmol g_{DW}^{-1} s^{-1}) predicted for hearts containing 0% mito-CK heart in Perry et al. (1988b), using the relationship between ATP production and CK flux to determine V_{max}. However, if the in vitro constants are used to describe the work-state kinetics, the V_{max} is 40.5 μmol g_{DW}^{-1} s^{-1}. There are no experimental data to suggest the V_{max} for hearts lacking mito-CK is this high. This comparison supports our conclusion that the dissociation constants obtained for MM-CK in solution accurately describe the KCl-arrested state, but not the working state. The CK flux of the KCl-arrested hearts is about $^1/_3$ of V_{max}, while that of the working hearts is $^2/_3$ of V_{max}.

A comparison of the rate equation constants for the working hearts with the NMR and biochemically determined substrate concentrations indicates that the CK reaction in the working neonatal heart is operating near saturation; that is, the substrate concentrations are at least 2-fold greater than the corresponding K_m or K_{im}. This is not the case in the KCl-arrested hearts studied, as the creatine concentration is well below its K_m. The other conclusion is that the enzyme complexes are more likely to yield product during isovolumic work than during KCl arrest, a finding supported by the NMR-derived rate constants.

The data do not permit distinction between changes in activation energy or "frequency factor" as the cause of the increased NMR-measured rate constants with work; decreased activation energy with work would require a change in the active-site geometry, perhaps induced by phosphorylation.

In summary, this study demonstrates that for hearts containing different guanidino substrate concentrations, in the absence of the mitochondrial CK isoenzyme, the NMR-derived rate constants are a function of cardiac work, whereas the absolute unidirectional flux is affected by substrate concentration and work state. The forward and reverse flux values are equal at moderate workloads. The mitochondrial CK isoenzyme is not a necessary condition for the change in NMR-determined rate constant, K_f, with transition from KCl arrest to work. The CK reaction in the intact heart appears rate limited in the direction of MgATP synthesis. The rate equation used to describe in vitro kinetics cannot, in any form, accurately describe the kinetics of the intact neonatal rabbit heart unless two sets of Michaelis and binary dissociation constants are specified. The MM-CK solution-derived constants appear to describe the KCl-arrested state, while the second set of constants determined from our experimental data describes the isovolumic work state.

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APPENDIX

The derivation of the rate equation used in the present study is given below. The reaction scheme at pH 8 (Figure 4) is similar to that shown in Scheme 1 of Morrison and James (1965) with the elimination of the E–MgADP form of the enzyme as noted in the text. The scheme at pH 7 is similar to that of Figure 9 of Schimerlik and Cleland (1973) with the same modification noted above. The assumption implicit in the derivation is that the reaction is a rapid equilibrium type; that is, the transition of the central complex is the rate-limiting step.

The various constants can be defined in terms of the reactants and products, which include both enzyme forms and the substrates participating in the CK reaction. By use of the convention of Cleland (1967), A = [MgADP], B = [PCr], P = [creatine], and Q = [MgATP].

The dissociation, Michaelis, and inhibition constants are defined as follows:

$$\boldsymbol{K}_{ia} = (E)A/(EA) \quad K_a = (EB)A/(EAB)$$
$$\underline{K}_{ip} = (E)P/(EP) \quad K_p = (EQ)P/(EQP)$$
$$\boldsymbol{K}_{ib} = (E)B/(EB) \quad K_b = (AE)B/(EAB)$$
$$K_{iq} = (E)Q/(EQ) \quad \underline{K}_q = (EP)Q/(EPQ)$$
$$K_{Ib} = (EQ)B/(EBQ) \quad K_{Ip} = (EA)P/(EAP)$$
$$K_{Iq} = (EB)Q/(EBQ) \quad (1)$$

The expressions for K_{ip} and K_q are underlined to indicate that they only apply at pH 8 for the conditions used by Morrison and Cleland (1965) and not at pH 7 as shown by Schimerlik and Cleland (1973). The expression for K_{ia} is in bold because it will not appear in the equation formulation because of substrate considerations (see text). The enzyme forms are shown in parenthesis.

The reaction, in the direction of ATP synthesis, can be expressed as

$$v = k_1{}^*(\mathrm{EAB}) \quad (2)$$

K_1 is defined as

$$k_1 = V_{\max}/E_t \quad (3)$$

where E_t is the sum of all enzyme forms present in the reaction scheme. Using the constraint set by substrate concentrations, E_t in the intact heart is given by the expression

$$E_t = E\left(1 + \frac{\mathrm{B}}{K_{ib}} + \frac{Q}{K_{iq}} + \frac{AB}{K_a K_{ib}} + \frac{QP}{K_{iq} K_p} + \frac{BQ}{K_{Ib} K_{iq}}\right) \quad (4)$$

By substitution of eq 4 into eq 3 and then eq 3 into eq 2 the rate equation for the pH 8 case is

$$v = V_{\max} AB/K_{ib} K_a (E_t/E) \quad (5)$$

The expression in eq 5 was used to calculate estimates for $V_{\max}$ by using the CK forward flux as measured by NMR saturation transfer as the value of v.

The dependence of $V_{\max}$ on [PCr] can be found by taking the partial differential of eq 5 with respect to B at constant A, P, and Q. The resultant is a constant indicating that $V_{\max}$ is linearly dependent on [PCr].

A summary of the constants used in the rate equations is given in Table VI.

Registry No. CK, 9001-15-4; PCr, 67-07-2; ATP, 56-65-5; ADP, 58-64-0; KCl, 7447-40-7; creatine, 57-00-1.

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