Adenosine Triphosphate Compartmentation in Living Hearts: A Phosphorus Nuclear Magnetic Resonance Saturation Transfer Study†

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ABSTRACT: ^{31}P nuclear magnetic resonance (NMR) studies of creatine phosphokinase (CPK) kinetics using saturation transfer techniques are reported. The phosphocreatine (PCr) and adenosine triphosphate (ATP) levels in perfused hearts can be altered experimentally by stopping the flow of perfusate (ischemia) to the heart for 35-min periods, followed by reperfusion to produce stable levels of performance. Utilization of energy by the heart was altered by administration of 25 mM potassium chloride (KCl) in the perfusate, which arrests contraction of the myocardium. Compared with control heart studies, the unidirectional rates measured during ischemia and KCl arrest are altered. The rates observed in the control experiments indicate that the CPK system is not in a steady state. This apparent deviation from steady-state conditions is ascribed to the existence of intracellular compartmentation of ATP.

Over the past 15 years there has been a steady accumulation of evidence for the compartmentation of pools of high-energy phosphate substrates in cardiac tissue. The following are the principal elements of this evidence: (1) the discovery of a mitochondrial-bound creatine phosphokinase (CPK[1]) (Jacobs et al., 1964) distinct from a cytosolic enzyme fraction, (2) the demonstration that the addition of creatine and ^{32}P-labeled ATP to heart mitochondria suspensions results in the production of PCr from the mitochondrial ATP but not from the exogenous ATP (Yang et al., 1977), (3) the additional demonstration that heart mitochondrial CPK phosphorylates creatine at a high rate even in the presence of a high concentration of PCr (Saks et al., 1975), and (4) the observation that the application of large concentrations of creatine to cardiac cells grown in culture produced large increases in PCr concentrations with no depletion in the cellular ATP levels (Seraydarian et al., 1974). Compartmentation is also supported by the observations that cardiac function is significantly impaired during fatigue and periods of nonperfusion (ischemia) when PCr levels fall markedly but ATP levels remain unchanged or relatively high (Dhalla et al., 1972).

In order to explain the apparent paradox that ATP, the prime source of free energy in muscle tissues, is restored by rephosphorylation from PCr while the maintenance of PCr levels must be accomplished by ATP, we invoked compartmentation. Clearly, CPK catalyzes these reactions. However, such a system can operate effectively only if the forward and reverse events are separated in space. Seraydarian & Abbott (1976) have presented a clear argument for this proposition.

We report in this paper a study of the chemical exchange processes for the PCr and γ-phosphate of ATP catalyzed by CPK in perfused rabbit hearts in normal, control experiments and in hearts reperfused following 35 min of total ischemia. The results from control experiments directly support the compartmentation hypothesis, and the results from the ischemic heart experiments indicate a loss of one pool of ATP and the establishment of a near-steady-state condition for the CPK reaction.

Chemical exchange processes in solutions have been examined by a variety of nuclear magnetic resonance (NMR) methods (Forsen & Hoffman, 1963; Campbell et al.. 1977). Saturation transfer has been employed in studies of conformational changes of molecules of biological interest and electron and proton exchanges in proteins (Dahlquist et al., 1975; Glickson et al., 1974; Gupta & Redfield, 1970). There have been several cogent reviews of the experimental and theoretical aspects for application of NMR techniques in the determination of rates of chemical exchange reactions (Johnson, 1965; Campbell et al., 1977, 1978). Brown & Ogawa (1977) have examined an in vitro system to measure the unidirectional rates of catalyzed exchanges in an adenylate kinase system and have applied similar techniques to in vivo measurements of the ATPase reaction in *Escherichia coli* (Brown et al., 1977) and the creatine kinase reaction in perfused rat hearts (Brown et al., 1978). We have applied a Fourier transform (FT) double-irradiation technique to the study of the creatine kinase reaction in normal perfused rabbit hearts which are performing isometric contractions against a fluid-filled balloon and in hearts prepared in an identical manner which have been subjected to 35 min of ischemia and then reperfused to a state of stable performance before making rate measurements.

Fourier transform techniques applied to NMR provide, in addition to significant time reductions in signal averaging, the added benefit of multiple pulse and multiple irradiation methods for the direct measurement of exchange rates. One such method, saturation transfer, is of general utility and is easily implemented.

Briefly, consider a simple chemical exchange system such that a slow reversible molecular process transfers a ^{31}P nucleus back and forth between two molecular environments, A and B. The disappearance of signal from site A results from the chemical exchange of A to B and from the longitudinal relaxation rate ($1/T_1$) of A. If site B is selectively irradiated so that the NMR signal of B is saturated, then there can be no contribution to the signal intensity of site A from the

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[1] Abbreviations used: CPK, creatine kinase; PCr, phosphocreatine; ATP, adenosine triphosphate; NMR, nuclear magnetic resonance; FT, Fourier transform; KCl, potassium chloride; γ-ATP, γ-phosphate of ATP; β-ATP, β-phosphate of ATP; T_1, longitudinal relaxation time; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; Mn^{2+}, manganese ion; Mg^{2+}, magnesium ion.

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transfer of nuclei from B to A. If the longitudinal relaxation rate is small (T_1 is long) relative to the rate of transfer of nuclei out of site A, it will be possible to measure the lifetime of nuclei in site A. The exact converse argument applies to the measurement of the rate for B exchanging to site A.

We have applied the saturation transfer technique to the PCr/ADP–ATP/Cr system in rabbit hearts which is equilibrated by creatine kinase using ^{31}P NMR to observe the major phosphorus-containing substrates in this phosphotransferase reaction. By this technique rates of chemical exchange between species are determined by observing the transfer of unsaturated spins out of one site and into the concomitant saturated site.

The results reported herein are the first in vivo studies to support the hypothesis of cellular compartmentation of ATP. Altered CPK kinetics for altered physiological states are also demonstrated.

Materials and Methods

Materials. Hearts were obtained from New Zealand white rabbits weighing 2.5–3.5 lb. Creatine phosphokinase (Type 1) isolated from rabbit muscle was obtained from Sigma Chemical Co. and used without further purification. Chelex-100 (100–200 mesh) was obtained from Bio-Rad Laboratories.

Methods. The isolated hearts were perfused by the method of Langendorf. Heart rate and left ventricular developed pressure were monitored by a small fluid-filled balloon placed in the left ventricle. The balloon was connected to a pressure transducer by a hydraulic line. The transducer output was amplified and fed to a strip chart recorder. Complete details have been published elsewhere (Hollis et al., 1978). For experiments involving ischemia, the flow of perfusate to the heart was stopped for 35 min. At the end of this time the flow was resumed and the heart was allowed to recover to a consistent level of performance.

Solutions of rabbit muscle CPK were made in Hepes–acetate buffer by using deionized, distilled water with the pH adjusted to 7.2. All substrates were dissolved in buffer and deionized with Chelex-100. Magnesium was added as the acetate salt. Prior to the addition of exogenous Mg^{2+}, an equilibrated CPK system showed no measurable rates. The chemical shift of the β-phosphate of ATP indicated little or no Mg-ATP (Cohn & Hughes, 1962), the necessary substrate for the CPK reaction. Addition of an amount of Mg^{2+} equal to the ATP concentration resulted in immediate establishment of maximum rates, and the concomitant 2-ppm downfield chemical shift of the β-ATP resonance was observed.

All spectra were obtained in the Fourier transform mode by utilizing a Bruker WH-180 spectrometer with a wide-bore 42.5-kG superconducting magnet. The isolated perfused hearts were placed in the 25-mm NMR tube, and all requisite lines were secured to a rod attached to the NMR tube. All spectra are the result of time averaging for 150 accumulations, a cycle time of 4 s, a broad-band pulse corresponding to a 70° flip angle, a sweep-width of 3 kHz and broad-band proton decoupling. Integrals used for the ATP/PCr ratios were corrected for the nuclear Overhauser effect (NOE). On perfused rabbit hearts the measured NOE's are 0.44 for PCr and 0.35 for γ-ATP. The resonance frequency for ^{31}P at the specified field is 72.88 MHz. Saturation of individual resonances in the spectrum was accomplished by applying a selective pulse of varying length from a laboratory-made homonuclear decoupler. The power output of this unit has been adjusted to provide adequate selective saturation without perturbation to adjacent signals by monitoring the effect of the saturation pulse

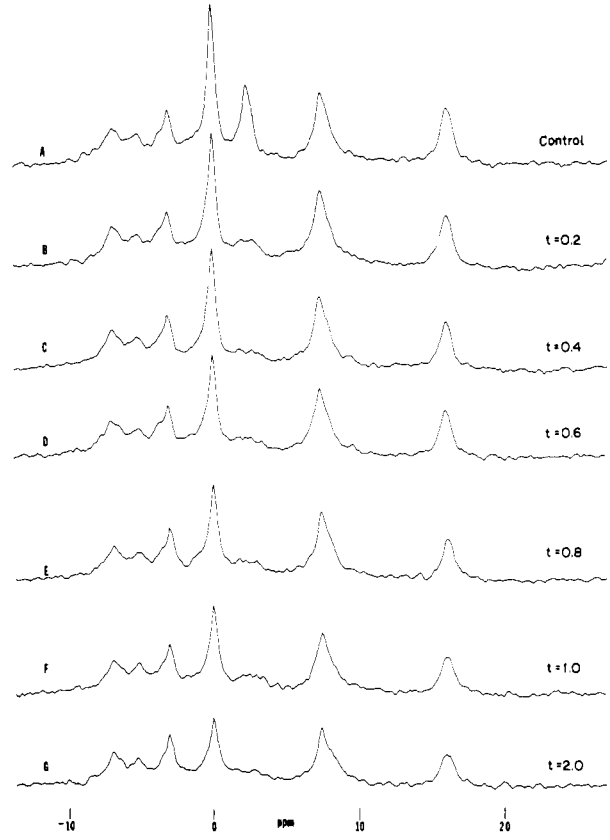

FIGURE 1: A series of control spectra of a normally perfused rabbit heart. Spectrum A is a control with the homodecoupler offset +5 ppm from PCr, and spectra B–G are the results of applying a saturating pulse for the indicated interval at the γ-ATP resonance frequency.

when offset 100–200 Hz from resonances of interest. Campbell et al. (1978) have explained this double-resonance FT method in detail. Secondary control spectra were obtained with the homonuclear decoupler offset 500–1000 Hz from the resonances of interest and subtracted from the first controls (homodecoupler offset by 100–200 Hz) to monitor the selectivity of the pulse in each heart experiment. Perturbations of PCr signal intensities of <3% were obtained for homodecoupler pulses of 50 ms and a decoupler offset of 150 Hz. No consistent changes in intensities were found for an identical offset value with decoupler pulses of 100 ms or longer. All chemical shifts are given in parts per million relative to PCr. The field stability of the superconducting magnet allowed spectra to be obtained without a field-frequency lock.

Results

A typical series of control spectra are shown in Figure 1. In order to obtain the individual pseudo-first-order rate constants, we used the general expressions

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \tag{1}$$

$$\frac{M^A{}_0}{M^A{}_{t\to\infty}} = \frac{T_{1(A)}}{\tau_{1(A)}} \tag{2}$$

$$\frac{1}{\tau_{1(A)}} = \frac{1}{T_{1(A)}} + k_1^* \tag{3}$$

Equation 1 defines a simple two-component chemical exchange system. Equation 2 describes the relationship of the NMR signal intensity arising from the A site for two different equilibrium states. $M^A{}_0$ is the intensity of the signal from A

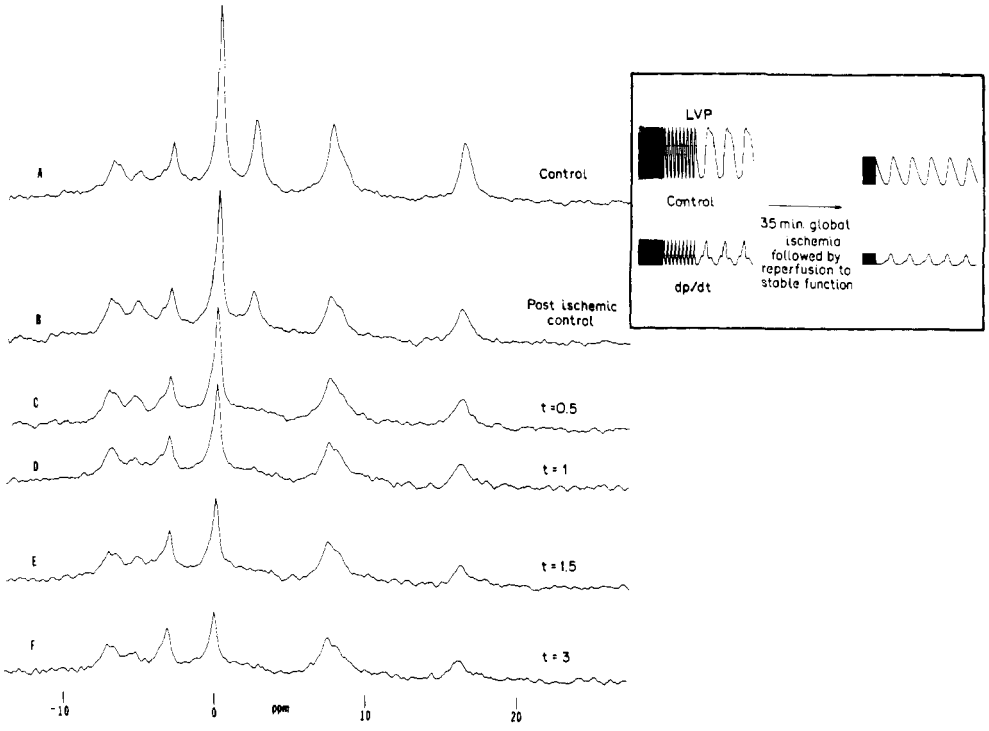

FIGURE 2: A series of rabbit heart spectra showing the initial control (A) and subsequent reperfusion spectrum (B) following 35 min of ischemia and approximately 12 min of reperfusion to a condition of a stable performance. Spectra C–F are the results of the selective saturation of the γ-ATP resonance as described in Figure 1. The box in the upper right-hand corner shows the performance of this heart before and after ischemia.

in the absence of any saturation of the B site. $M^A_{t\to\infty}$ is the final residual A intensity after a long period of saturation of B; any increased duration in the saturation time produces no further diminution in the A intensity. The ratio of the initial and final integrated signals from A gives the value for $T_{1(A)}/\tau_{1(A)}$. A plot of ln $(M^A_t - M^A_{t\to\infty})$ vs. time (t = the particular interval of application of the selective saturating irradiation) yields a straight line having a slope of $-1/\tau_{1(A)}$ (Forsen & Hoffman, 1963; Campbell et al., 1977). The rate k_1* is calculated directly by using eq 3 once the slope of the ln/linear plot is found. T_1 is the longitudinal relaxation time of the A species and k_1*, in this example, is the forward pseudo-first-order rate of the A $\rightleftarrows$ B reaction.

Figure 2 shows a series of spectra from a recovered ischemic heart in which γ-ATP is saturated. In the ischemia experiments, the saturation of the PCr resonance gives a plot of the γ-ATP intensities for which there is a marked alteration in the slope which is reflected in both the T_1 and k_1* values obtained from these experiments (Figure 3). In a previous report, 30–40-min periods of ischemia, followed by reperfusion, produced large alterations in the ratio of PCr to γ-ATP in ^{31}P spectra of rabbit hearts relative to spectra obtained under normal, control conditions (Hollis et al., 1978). Without kinetic data, however, no clear understanding of what is occurring in the exchange system can be made.

Similar measurements were made on a heart that had been arrested by addition of 25 mM KCl to the perfusate. In this case the k_1* was 0.15 s^{-1} and the k_2* was 0.15 s^{-1} also. The marked decrease in rates for the arrested heart as compared to a beating heart is consistent with the idea that the turnover rate of the CPK reaction is coupled to the energy demands of the heart. The results also show for the first time a correlation between the in vivo rate of an enzymatic reaction and the physiological state of the heart.

The two important findings are the marked increase, relative to control conditions, in the forward rate of the creatine kinase

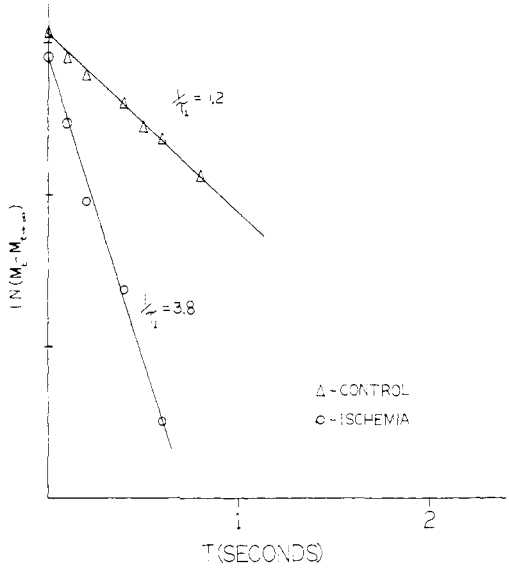

FIGURE 3: Typical plots of the saturation transfer experimental data for a normally perfused heart and a recovered ischemic heart. The slope of each plot is equal to $-1/\tau_1$. These data are the result of saturating the PCr resonance and are used to calculate T_1 of γ-ATP and k_1*.

reaction and a significant decrease in the T_1 of the γ-ATP. In these experiments the k_1* increases by nearly 2.5-fold and the T_1 decreases by 2-fold when compared with control values. To compare the rates to determine if the system is at a steady state, we used the equations

$$k_1^*[\text{ATP}] = k_2^*[\text{PCr}] \quad (4)$$

$$k_1^*\frac{[\text{ATP}]}{[\text{PCr}]} = k_2^* \quad (5)$$

and k_1* and k_2* are the measured pseudo-first-order rates from the saturation transfer experiments.

Table I

	k_1* (s^{-1})[b]	γ-ATP T_1[b]	k_2* (s^{-1})[b]	PCr T_1[b]	PCr/γ-ATP[a,b]	k_1*([ATP]/[PCr]) (s^{-1})[b]
control	0.52 ± 0.1	1.2 ± 0.1	0.70 ± 0.1	1.8 ± 0.1	1.2 ± 0.2	0.43
ischemia experiments	1.44 ± 0.3	0.6 ± 0.1	0.74 ± 0.06	2.2 ± 0.4	2.0 ± 0.3	0.72
KCl arrest	0.15	1.25	0.15	2.3	1.35	0.11

[a] Corrected for nuclear Overhauser effect (NOE) on PCr and γ-ATP. [b] The mean and standard deviations were calculated on four or five separate determinations except for the KCl arrest data. All rates are in units of s^{-1}.

For the normal control experiments the result is k_1*([ATP]/[PCr]) = 0.43 s^{-1} compared with k_2* = 0.70 s^{-1}, which indicates clearly a system not at steady state. The corresponding results for the reperfused, ischemic experiments are k_1*([ATP]/[PCr]) = 0.72 s^{-1} compared with k_2* = 0.74 s^{-1}. Table I is a complete compilation of all data.

As a further check of the measurements, rates were measured for a CPK system in solution using purified rabbit muscle CPK at a concentration of 5 mg mL^{-1}. When the PCr/ATP ratio was 1, the rates were k_1* = 0.26 s^{-1} and k_2* = 0.27 s^{-1}. Readjusting the PCr/ATP ratio in solution to 1.5, we found that the measured rates were k_1* = 0.44 s^{-1} and k_2* = 0.31 s^{-1}; multiplication of k_1* by the ATP/PCr ratio yields the value of 0.29 s^{-1}.

Discussion

The kinetic data offer a direct confirmation of the compartmentation of cellular pools of ATP. We make this conclusion on the basis that in control experiments the CPK reaction appears not to be in a steady state as determined from the comparison of the forward and reverse rates which should be equal in the steady state according to eq 4. Additional support for compartmentation comes from unpublished NMR experiments (Bulkley et al.) utilizing manganese (Mn^{2+}) as a paramagnetic probe for metal ion transport. In the latter experiments the obliteration of ^{31}P resonances from the heart is measured as a function of time after administration of low levels of Mn^{2+} via the perfusate. A rapid, initial loss of nearly all the PCr and part of the ATP intensities is observed. A fractional amount of the ATP resonances takes much longer (2–3 times) to disappear. This must be an ATP pool not as accessible to Mn^{2+} as the cytosolic pool.

Based on the long-term maintenance of ^{31}P-containing substrates and the stable performance of isolated perfused hearts, the clear implication is that the CPK systems are in a steady state. The kinetic results appear to contradict this simply because we cannot multiply the k_1* values by the proper ATP/PCr ratios. There may be multiple intracellular ATP compartmentation, but in the case of the CPK measurements there appear to be two major ATP pools. The problem can be illustrated by using a two-compartment model

$$\mathrm{ATP} + \mathrm{Cr} \underset{k_2^{A}}{\overset{k_1^{A}}{\rightleftharpoons}} \mathrm{PCr} + \mathrm{ADP} + \mathrm{H}^+$$

compartment A

$$\mathrm{ATP} + \mathrm{Cr} \underset{k_2^{B}}{\overset{k_1^{B}}{\rightleftharpoons}} \mathrm{PCr} + \mathrm{ADP} + \mathrm{H}^+$$

compartment B

and

$$k_1^{A}\frac{[\mathrm{ATP}]_A}{[\mathrm{PCr}]_A} = k_2^{A}$$

$$k_1^{B}\frac{[\mathrm{ATP}]_B}{[\mathrm{PCr}]_B} = k_2^{B}$$

Since the present NMR results do not discriminate the substrate concentrations in any compartment, the measured k_1* and k_2* values are compared on the basis of the ATP/PCr ratio obtained from the entire heart.

The indication of near-steady-state kinetics in the reperfused ischemic heart experiments may result simply from the loss of ATP from one compartment. The decrease in T_1 for the γ-phosphate of the remaining ATP is also consistent with the selective loss of one pool of ATP. It may be a mitochondrial pool of ATP which remains since this would have a shorter T_1 due to the high protein and membrane content of these organelles. In the control experiments the longer T_1 obtained for γ-ATP results from the averaging of a longer T_1 component with the shorter one.

We recognize that there are other avenues of chemical exchange which could also influence the measurement of k_1* in these studies, since the γ-phosphate of ATP is cycled through many other reactions. However, as the saturation of γ-ATP for long intervals produces no measurable changes in the sugar phosphate and orthophosphate resonances in these spectra and only small changes in the β-ATP resonance (adenylate kinase reaction), such contributions are evidently small. Even if other exchanges are occurring, were all the exchanging systems in a steady state, the measured rates for any one system should still produce a result consistent with steady-state conditions.

In summary, the application of saturation transfer techniques to perfused hearts for the purpose of measuring "in vivo" kinetics of the creatine kinase reaction is extended to the case for recovered hearts following ischemic intervals which produce new PCr/γ-ATP ratios. The particular type of experiment used permits the determination of both the T_1 and rate constant for any directly observable species involved in a measurable exchange. The results directly support the hypothesis of compartmentation of ATP which can explain the apparent non-steady-state condition for the creatine kinase reaction. It is clear that other such studies may further elucidate important aspects of cardiac physiology. At present we are applying saturation transfer techniques in the study of altered physiological states of the heart and the metabolic effects of drugs on specific enzymic reactions to gain further insight into cellular compartmentation of substrates and the implications of such compartmentation. Further, the application of such measurements in localized volumes of perfused hearts utilizing NMR imaging methods could provide new information on the effects and viability of tissue in regions of acute infarction.

Acknowledgments

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