erican Chemical Society, Chicago, Sept 8–13, 1985, American Chemical Society, Washington, D.C.
Shortle, D. (1983) *Gene 22*, 181.
Shortle, D., & Lin, B. (1985) *Genetics 110*, 539.
Sillen, L. G., & Martell, A. E. (1964) *Spec. Publ.–Chem. Soc. 17*.
Slater, J. P., Tamir, I., Loeb, L. A., & Mildvan, A. S. (1972) *J. Biol. Chem. 247*, 6784.
Tucker, P. W., Hazen, E. E., Jr., & Cotton, F. A. (1978) *Mol. Cell. Biochem. 22*, 67.
Tucker, P. W., Hazen, E. E., Jr., & Cotton, F. A. (1979) *Mol. Cell. Biochem. 23*, 3.

^{31}P NMR Saturation Transfer Measurements of Phosphorus Exchange Reactions in Rat Heart and Kidney in Situ†

Alan P. Koretsky,‡,§ Samuel Wang,‖ Melvin P. Klein,§ Thomas L. James,⊥,# and Michael W. Weiner*,‡,‖,#
Medical Service, Veterans Administration Medical Center, San Francisco, California 94121, Chemical Biodynamics Division, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720, and Departments of Medicine, Pharmaceutical Chemistry, and Radiology, University of California, San Francisco, California 94133
Received February 19, 1985; Revised Manuscript Received July 29, 1985

ABSTRACT: ^{31}P NMR spectra of rat kidney and heart, in situ, were obtained at 97.2 MHz by using chronically implanted radio-frequency coils. Previous investigators have used magnetization transfer techniques to study phosphorus exchange in perfused kidney and heart. In the current experiments, saturation transfer techniques were used to measure the steady-state rate of exchange between inorganic phosphate (P_i) and the γ-phosphate of ATP (γATP) in kidney, and between phosphocreatine (PCr) and γATP, catalyzed by creatine kinase, in heart. The rate constant for the exchange detected between P_i and γATP in kidney, presumably catalyzed by oxidative phosphorylation, was 0.12 ± 0.03 s^{-1}. This corresponds to an ATP synthesis rate of 12 μmol min^{-1} (g wet weight)$^{-1}$. Comparison of previously published O_2 consumption and Na^+ reabsorption rates for the intact kidney with the NMR-derived rate for ATP synthesis gave flux ratios of $J_{ATP}/J_{O_2} = 1.6$–3.3 and $J_{Na^+}/J_{ATP} = 4$–10. The rate constants for the creatine kinase reaction, assuming a simple two-site exchange, were found to be 0.57 ± 0.12 s^{-1} for the forward direction (PCr → ATP) and 0.50 ± 0.16 s^{-1} for the reverse direction (ATP → PCr). The forward rate (0.78 ± 0.18 intensity unit/s) was significantly larger ($p < 0.05$) than the reverse rate (0.50 ± 0.16 intensity unit/s). This difference between the forward and reverse rates of creatine kinase has been previously noted in the perfused heart. The difference has been attributed to participation of ATP in other reactions. To test this possibility, the reverse rate was measured by saturating both PCr and the β-phosphate of ATP. The rate obtained in this manner was not significantly different from that derived by assuming a two-site exchange. These results suggest the possibility that compartmentation of ATP might be responsible for the difference in rates.

A detailed understanding of the regulation and control of metabolic pathways requires an estimiate of the rates of each individual reaction. For the case of the generation of ATP by oxidative phosphorylation and utilization for various work functions (e.g., Na^+ transport and muscle contraction), most kinetic information comes from in vitro studies of isolated subcellular systems (Hansford, 1980). However, functioning of regulatory mechanisms in vivo may be altered in vitro. Physiologic experiments performed on intact tissue have produced information concerning the coupling of oxidative metabolism to work demands (Mandel & Balaban, 1981). These studies have had to rely on assumptions concerning detailed reaction mechanisms, such as the stoichiometry of ATP formation from oxidative phosphorylation. To bridge the gap between in vitro studies of isolated mitochondria and physiologic studies, it is important to know the rate of metabolic reactions, such as ATP formation and utilization, in vivo.

NMR techniques can be used to measure the rates of enzyme reactions in vivo (Brown et al., 1977; Gupta, 1979). In particular, by use of ^{31}P NMR magnetization transfer techniques, the rate of the creatine kinase reaction has been measured in perfused heart (Brown et al., 1977; Matthews et al., 1982; Nunally & Hollis, 1979), isolated muscle (Brown et al., 1979; Gadian et al., 1981), and rat and turtle brain in situ (Balaban et al., 1983; Shoubridge et al., 1982; Wemmer et al., 1982). Additionally, the rate of ATP synthesis has been measured in the perfused heart and kidney (Freeman et al., 1983; Matthews et al., 1981; Yahaya et al., 1984) as well as the rat brain (Shoubridge et al., 1982) and maize root tips (Roberts et al., 1984).

Techniques have been developed in this laboratory which allow ^{31}P NMR spectra to be obtained from rat kidney, heart, and liver in situ (Weiner et al., 1980; Koretsky et al., 1982, 1983). The stability of the animals and the high metabolic rates which occur in these organs in situ offer advantages over

† This work was supported by National Institutes of Health Grant AM 3392-01A1, the Veterans Administration Medical Research Services, the Research and Education Allocation Committee and Faculty Senate Research Committee of U.C.S.F., the Hedco Foundation, the Northern California Heart Association, and the Office of Energy Research, Office of Health and Environmental Research, Health Effects Research Division of the U.S. Department of Energy, under Contract DE-AC03-765-F00098.
* Address correspondence to this author at the Veterans Administration Medical Center, University of California, San Francisco.
‡ Veterans Administration Medical Center.
§ University of California, Berkeley.
‖ Department of Medicine, University of California, San Francisco.
⊥ Department of Pharmaceutical Chemistry, University of California, San Francisco.
Department of Radiology, University of California, San Francisco.

0006-2960/86/0425-0077$01.50/0

perfused organs for magnetization transfer studies. The results of the experiments reported here represent the first in situ measurements of the rates of exchange between the phosphorus moiety of phosphocreatine (PCr) and ATP in heart and between P_i and ATP in kidney.[1]

Materials and Methods

Surgical Procedure. Details of animal methods for NMR experiments have been described previously (Koretsky et al., 1983). Female Sprague-Dawley rats (180–220 g) were used throughout. Two-turn, radio-frequency (rf) coils were made from 22-gauge copper wire and insulated with polyethylene tubing (Clay-Adams, PE-100). Small ceramic chip capacitors (American Technical Ceramics) were placed close to the coil to partially tune and match the circuit, as well as minimize losses associated with implantation (Murphy-Boesch & Koretsky, 1983). These coils were surgically implanted around the heart or kidney, 3–5 days prior to NMR experiments. Two days prior to implantation of the kidney coil, rats were uninephrectomized.

On the day of NMR experiments, animals were kept anesthetized with a continuous dose of a Ketamine/Rompun mixture, administered through an intraperitoneal line. A venous line for administering saline and an arterial line to monitor blood pressure were also used. Just prior to kidney experiments, the rats were volume-expanded (4% of body weight) over the course of 30 min with freshly prepared plasma.

NMR Measurements. All measurements were performed at a phosphorus frequency of 97.2 MHz in a 3-in.-diameter vertical bore superconducting magnet (Cryomagnets Corp.) equipped with a home-built spectrometer and a Nicolet 1180/293B data system.

Rats were placed in a specially designed holder, and the coil leads were soldered to the remainder of the tuning and matching circuit. The coil was tuned to the appropriate frequency and the holder placed in the magnet so that the organ was centered in the magnetic field. For the kidney experiments, this meant securing the rat in an upside-down position because the spinner assembly prevented placing the rat head up. The ^1H signal from water in the organ was used to shim the magnetic field before phosphorus spectra were obtained. No field frequency lock was employed.

Saturation transfer experiments were performed following Forsén & Hoffman (1963). The unidirectional exchange of phosphate from ATP to PCr is used to briefly describe the technique. The PCr resonance was selectively saturated long enough so that the intensity of the γ-phosphate of ATP (γATP) peak reached a steady state ($M^{ss}_{\gamma ATP}$). This steady-state level was compared to the equilibrium magnetization of γATP ($M^0_{\gamma ATP}$) by obtaining a spectrum with the selective rf irradiation at a control frequency displaced an equal distance as the PCr resonance from the γATP signal but on the upfield side. To minimize the effects of any biological variations, control and saturated spectra were obtained concurrently by alternating eight pulses with the rf power in each position.

The apparent T_1 ($T_{1\,app}$) of the γATP resonance was then measured by using saturation recovery techniques (McDonald & Leigh, 1973) with the PCr peak saturated throughout the measurement. Saturation of the whole spectrum was accomplished by a burst of 90° pulses of alternating phase (Matson et al., 1984) separated by 10-ms homospoil pulses. A sequence of time intervals between spectral saturation and observation of the γATP resonance, while maintaiing the PCr peak saturated, was used to construct a T_1 plot. A best three-parameter, single-exponential fit, performed on the spectrometer's Nicolet computer, was used to extract $T_{1\,app}$ from the data. That this method gave accurate T_1's was demonstrated by comparing the T_1 of methylenediphosphonic acid (MDPA) obtained from a capillary attached to a kidney coil in a rat to the T_1 of MDPA obtained from a conventional probe by using standard inversion recovery techniques. The two values agreed within experimental error.

The unidirectional rate constant for the reaction ATP → PCr ($k_{ATP\rightarrow PCr}$) was derived from the above measurements by using the expression

$$k_{ATP\rightarrow PCr} = \frac{M^0_{\gamma ATP} - M^{ss}_{\gamma ATP}}{M^0_{\gamma ATP} T_{1\,app}} \quad (1)$$

The rate of the reaction ATP → PCr was obtained by multiplying $k_{ATP\rightarrow PCr}$ by a suitable quantity of ATP expressed in arbitrary intensity units, obtained from the NMR spectra.

Specific spectral acquisition parameters are given in the figure legends. The pulse used to saturate specific peaks in the spectrum was generated by using the decoupler channel of the spectrometer. Typically, peak to peak voltages of from 0.1 to 0.3 V representing from 4 to 18 mG were employed to saturate the appropriate peak. The peak was considered fully saturated if it was not visible in the NMR spectrum.

Apart from an exponential filter of 30 Hz, applied to the free induction decay prior to Fourier transformation, no other data massage techniques, e.g., convolution difference or left shifting, were used. To quantitate peak areas, two procedures were used. For heart spectra, where the peaks were resolved and there was not much contribution from an underlying broad component, the Nicolet integration routine was employed. For kidney spectra, not all the peaks were well resolved, and there was a larger broad component making use of the integration routine difficult; therefore, the Nicolet simulation routine NTCCAP was used. All the peaks, including the broad component, were fit by varying position, intensity, and line width until a minimum root mean square residual was obtained. Comparison of the two procedures for heart spectra yielded the same results within experimental error. All errors are reported as ±1 standard deviation, and statistical significance was calculated by using the Student's t test.

Results

Figure 1 shows typical spectra obtained from kidney (a) and heart (b) by using chronically implanted coils. The peaks seen, their relative magnitudes, and the signal to noise ratios obtained are similar to spectra obtained from perfused organs. The small PCr peak in the kidney spectrum suggests that an insignificant signal from surrounding muscle contributed to the P_i and ATP peaks (Koretsky et al., 1983). Similarly, the PCr/ATP ratio of the heart spectrum was the same or lower than that reported for perfused heart (Matthews et al., 1981, 1982) and much lower than that seen in skeletal muscle (Gadian et al., 1981), indicating that the spectrum is largely from heart.

Kidney. Figure 2 shows the saturation transfer experiment performed on the rat kidney in situ. In this case, spectra with the γATP peak saturated (Figure 2b) and with the selective rf irradiation in the control position (Figure 2a) were alternated every 8 pulses until a total of 128 acquisitions were obtained. The arrows indicate the position of the selective rf field.

[1] Preliminary results of this work have been reported previously (Koretsky et al., 1983, 1984; Koretsky & Weiner, 1984).

Table I: Results of Saturation Transfer Experiments Performed on Rat Kidney in Situ

reaction	$(M^0 - M^{ss})/M^0$	$T_{1\,app}$[a] (s)	k (s^{-1})	T_1[b] (s)
$P_i \rightarrow \gamma ATP$	0.15 ± 0.04	1.24 ± 0.16	0.12 ± 0.03	1.46 ± 0.12
$\beta ATP \rightarrow \gamma ATP$	0.20 ± 0.08 (n = 8)	0.55 ± 0.07 (n = 3)	0.36 ± 0.14	0.69 ± 0.11

[a] $T_{1\,app}$ and T_1 values are for reactants on the left side of the equations. [b] T_1 is the intrinsic T_1 or the T_1 calculated to account for the effects of exchange.

Table II: Results of Saturation Transfer Experiments Performed on Rat Heart in Situ

reaction	$(M^0 - M^{ss})/M^0$	$T_{1\,app}$[a] (s)	k (s^{-1})	T_1[b] (s)
$PCr \rightarrow \gamma ATP$	0.56 ± 0.09	0.98 ± 0.13	0.57 ± 0.12	2.23 ± 0.11
$\gamma ATP \rightarrow PCr$	0.27 ± 0.06	0.54 ± 0.13	0.50 ± 0.16	0.74 ± 0.10
$\beta ATP \rightarrow \gamma ATP$	0.23 ± 0.04 (n = 7)	0.52 ± 0.09 (n = 3)	0.44 ± 0.11	0.68 ± 0.11

[a] $T_{1\,app}$ and T_1 values are for reactants on the left side of the equations. [b] T_1 is the intrinsic T_1 or the T_1 calculated to account for the effects of exchange.

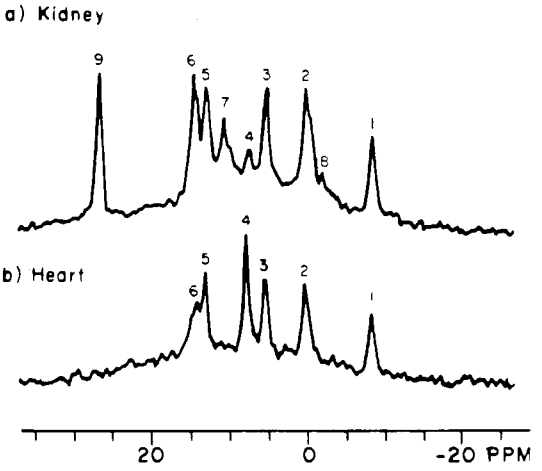

FIGURE 1: ^{31}P NMR spectra of (a) kidney and (b) heart in situ. Spectra are the sum of 128 acquisitions, using a 90° pulse and a 10-s recycle time. Numbers on peaks refer to (1) βATP, (2) αATP and NAD(H), (3) γATP, (4) PCr, (5) P_i, (6) phosphomonoesters, (7) phosphodiesters and urine P_i in kidney, (8) ?, and (9) the internal standard methylenediphosphonic acid.

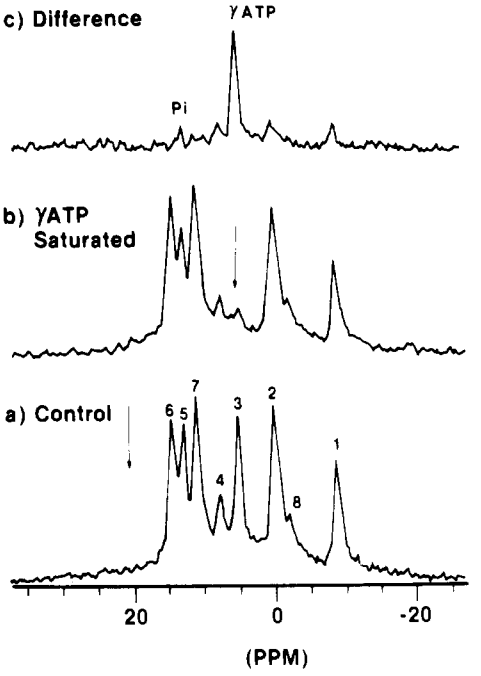

FIGURE 2: Saturation transfer measurement of the $P_i \rightarrow$ ATP reaction in rat kidney. Spectra a and b are sums of 128 acquisitions using a 90° pulse and a 10-s recycle time during which the saturation pulse was left on. Eight spectra were alternated with the rf irradiation on (a) the control frequency and (b) the γATP peak (arrows) until 128 acquisitions for each spectrum had been obtained. (c) Difference spectrum obtained by subtracting (b) from (a). Peaks are numbered as in Figure 1.

Comparison between spectra with no selective irradiation and spectra in which irradiation was applied in the control position indicated that the P_i peak was not significantly affected by stray irradiation from the saturation pulse. The saturation pulse did reduce the intensity of the broad component. To determine whether nonselective effects of saturating the broad component could produce artifacts resembling the effects of exchange, the broad component was saturated at various positions where there were no visible NMR peaks. Difference spectra using controls with no selective irradiation (Figure 1a) showed no changes in the magnetization of narrow resonances, although the broad component was reduced to aproximately 52% of its control value. This observation suggests a potential method for reducing the broad component associated with heart, kidney, and liver spectra. Recently, this approach has been used to reduce the broad component associated with bone when performing NMR of the brain (Ackerman et al., 1984; Gonzalez-Mendez et al., 1984).

The difference spectrum (Figure 2c) shows a reduction of the P_i magnetization when the γATP peak is saturated. Saturation of the P_i resonance had no effect on the γATP signal, possibly due to the short T_1 of the γATP resonance. Besides P_i, saturation of the γATP peak caused a reduction in both the αATP and βATP resonances. This was confirmed by the appropriate control experiments for these peaks. Saturation of the γATP peak can lead to a decrease in the βATP intensity by at least three mechanisms: (1) dipolar interactions between the γ- and β-phosphorus nuclei; (2) chemical exchange between the γ- and βATP catalyzed by two cycles of the adenylate kinase reaction (Gupta, 1979); or (3) simultaneous saturation of the nearby βADP resonance when the γATP peak is irradiated, which exchanges with βATP during reactions catalyzed by creatine kinase, ATPases, or adenylate kinase. The reduction in the αATP magnetization upon saturation of the γATP peak has no obvious explanation although it has been observed in experiments performed on rat brain (Shoubridge et al., 1982). Table I presents the experimental results which lead to a calculated rate constant, $k_{P_i \rightarrow ATP}$, of 0.12 ± 0.03 s^{-1}.

Heart. Figure 3 illustrates the results of an experiment performed to measure the rate constant for the reaction PCr → ATP ($k_{PCr \rightarrow ATP}$). Two spectra were obtained by alternating the selective rf power in the control position (Figure 3a) with the saturation pulse on the γATP signal (Figure 3b). The arrows indicate the positions of the rf field. The difference between these two spectra (Figure 3c) indicates the decrease in PCr magnetization resulting from exchange. From these

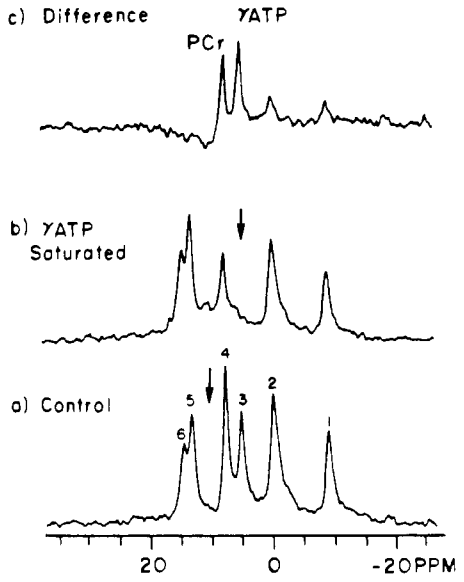

FIGURE 3: Saturation transfer measurement of the PCr → ATP reaction in rat heart. Spectra a and b are sums of 256 acquisitions each, using a 90° pulse and a 10-s recycle time during which the saturation power was applied. Eight spectra were alternated with the rf irradiation on (a) the control position and (b) the γATP position (arrows) until 256 acquisitions for each spectrum had been obtained. (c) Difference spectrum obtained by subtracting (b) from (a). Peaks are numbered as in Figure 1.

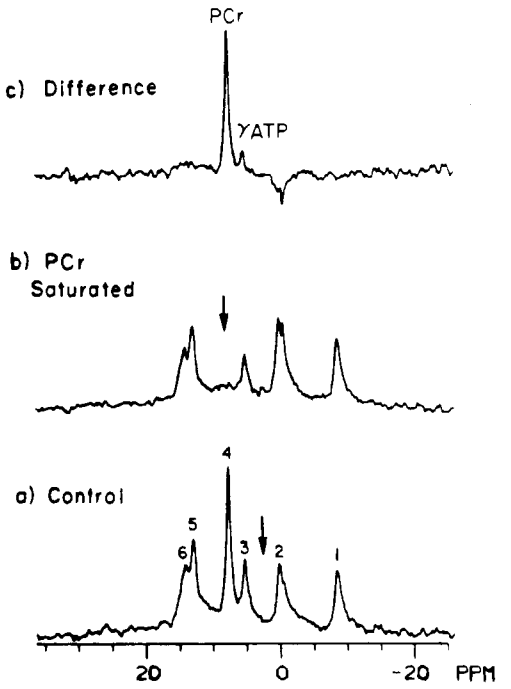

FIGURE 4: Saturation transfer measurement of the ATP → PCr reaction in rat heart. Experimental parameters were the same as those for Figure 3. Arrows indicate the position of the selective rf irradiation: (a) control; (b) PCr saturated; and (c) difference of (a) − (b). Peaks are numbered as in Figure 1.

measurements, a value of 0.56 ± 0.09 for $(M^0_{PCr} - M^{ss}_{PCr})/M^0_{PCr}$ was derived (Table II).

To assess the nonselective effects that saturation had on the magnetization of PCr, spectra without any selective irradiation (Figure 1b) were compared to spectra obtained with the rf field in the control position (Figure 3a). Irradiation reduced the PCr intensity to 88 ± 11% of its equilibrium value. Because the PCr peak appeared symmetrical, it was assumed that the effects of nonselective saturation were equivalent whether the selective rf pulse was in the control position or on the γATP resonance. As an indication that the value of $(M^0 - M^{ss})/M^0$ for PCr was not affected by nonselective irradiation, the γATP resonance was saturated to varying degrees from 0 to 100% by decreasing the power of the selective rf irradiation. At the lower power levels (γATP 0–50% saturated), there were no nonselective effects of saturation detected on the PCr magnetization. A value of $(M^0 - M^{ss})/M^0$ for PCr with γATP 100% saturated was extrapolated by using the values of $(M^0_{PCr} - M^{ss}_{PCr})/M^0_{PCr}$ with γATP partially saturated. This extrapolated value was equal, within experimental error, to that obtained with γATP saturated completely.

Similar to the kidney, the saturation pulse led to a 45% decrease of the broad component in heart, but no exchange between the broad component and narrow resonances was detected. Furthermore, saturation of the γATP peak lead to a reduction in the α- and βATP intensities (Figure 3c). Irradiation of the γATP peak had no effect on the P_i signal in contrast to results obtained in the perfused heart (Matthews et al., 1981). This could be due to low levels of intracellular P_i in the heart in situ, especially when compared to the large contribution of 2,3-diphosphoglycerate in blood to this region of the spectrum.

Figure 4 illustrates the saturation transfer experiment performed to measure the reverse rate, ATP → PCr, by saturating the PCr resonance and monitoring the reduction in γATP magnetization. As can be seen in the difference spectrum (Figure 4c), saturation of the PCr resonance perturbs only the γATP peak. The negative peak in the αATP region

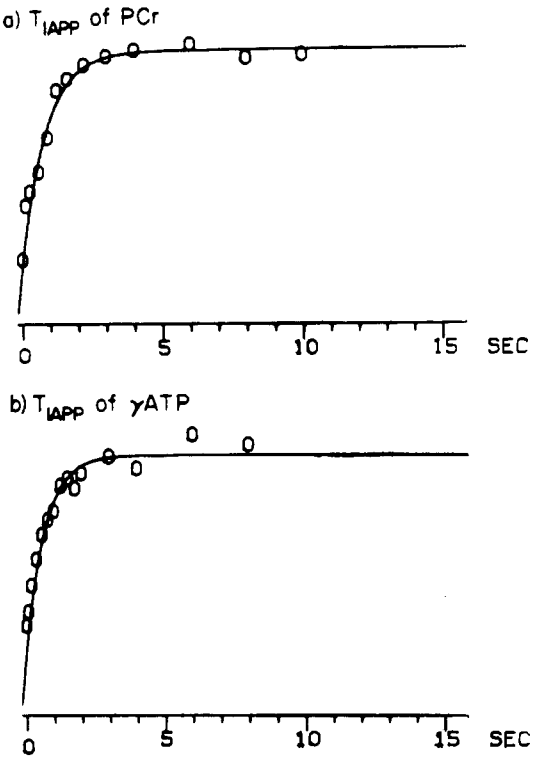

FIGURE 5: Data used for the determination of $T_{1\,app}$ of (a) PCr and (b) γATP in heart. Solid lines represent the best single exponential fit to the data points (open circles).

was due to nonselective effects that the control irradiation (arrow, Figure 4a) had on peaks in this region. Typical data to derive $T_{1\,app}$ for both the PCr and γATP peaks are presented in Figure 5.

Table II presents the experimental data and the calculated rate constants. For the forward direction of the creatine kinase reaction (PCr → ATP), a rate constant of 0.57 ± 0.12 s^{-1} was

found. A value of 0.50 ± 0.16 s^{-1} was measured for the reverse direction (ATP → PCr). Data are also included for the effect on the βATP peak.

To calculate the relative forward and reverse rates of the creatine kinase reaction, the rate constants derived above were multiplied by the concentrations of PCr and ATP expressed in arbitrary intensity units. The PCr/ATP ratio was 1.29 ± 0.13 (n = 7). The forward rate, PCr → ATP, was 0.74 ± 0.18 intensity unit/s, and the reverse rate, ATP → PCr, was 0.50 ± 0.16 intensity unit/s. The error of these determinations was considerable due to the use of three experimentally determined parameters and the inability to tightly control cardiac function in vivo. Nevertheless, these rates were significantly different ($p < 0.05$), in agreement with results previously reported for the perfused heart (Brown et al., 1979; Nunally & Hollis, 1979; Matthews et al., 1982; Kobayashi et al., 1982). Because creatine kinase is believed to be the only enzyme which utilizes PCr, and PCr appears to be in a steady state, it is impossible for the rate of PCr production to be larger than the rate of PCr utilization. Two explanations have been offered for this discrepancy between the measured forward and reverse rates. One possibility is that subcellular compartmentation of metabolites and/or enzyme renders the simple two-site exchange model, presented under Materials and Methods, inadequate (Nunnally & Hollis, 1979). Another possibility is that the participation of ATP in other reactions (e.g., ATPase, adenylate kinase) causes a spurious reduction of the rate (Brown et al., 1979). This would occur because nuclei from metabolites other than PCr are exchanging with γATP and have not been included in the two-site analysis.

To test for the involvement of competing reactions, all metabolites in the spectrum were successively saturated while monitoring the effect on the γATP resonance. If the γATP intensity was affected when a metabolite was saturated, this metabolite was considered to be involved in exchange with γATP to an extent comparable to PCR ⇄ γATP exchange. Saturation of the βATP peak caused a measurable change in the γATP peak; saturation of the other peaks (P_i, phosphomonoesters, αATP) had no effect on the γATP intensity. It has been suggested that exchange between PCr and γATP could mask the effects of competing exchanges (Ugurbil, 1984). To test this, the effect of saturating P_i on the γATP intensity was monitored while saturating PCr. Within the error of the measurements, neither the steady-state magnetization (n = 4) nor the $T_{1\,app}$ of γATP (n = 3) measured with PCr saturated was significantly affected by additional saturation of P_i.

These results indicate that the exchange between βATP and γATP was a major competing reaction which could affect the measurement of exchange between PCr and γATP. Interestingly, saturation of γATP affected the αATP resonance, but saturation of αATP had no effect on γATP. Reasons for this are not clear: however, it may indicate that the detected exchange with αATP is due to saturation of a small metabolite pool (e.g., βADP) which resonates near γATP.

The following expressions, derived from the Bloch equations, for the steady-state magnetization of γATP, in the presence of saturation of the PCr or βATP peaks, account for the additional exchange reaction between γ- and βATP:

$$\frac{M^{ss}{}_{\gamma ATP}}{M^{0}{}_{\gamma ATP}} = \frac{1 + (M^{ss}{}_{\beta ATP}/M^{0}{}_{\beta ATP})k_{\gamma ATP\rightarrow\beta ATP}T_{1\,\gamma ATP}}{1 + (k_{ATP\rightarrow PCr} + k_{\gamma ATP\rightarrow\beta ATP})T_{1\,\gamma ATP}} \quad (2)$$

$$\frac{M^{ss}{}_{\gamma ATP}}{M^{0}{}_{\gamma ATP}} = \frac{1 + (M^{ss}{}_{PCr}/M^{0}{}_{PCr})k_{ATP\rightarrow PCr}T_{1\,\gamma ATP}}{1 + (k_{ATP\rightarrow PCr} + k_{\gamma ATP\rightarrow\beta ATP})T_{1\,\gamma ATP}} \quad (3)$$

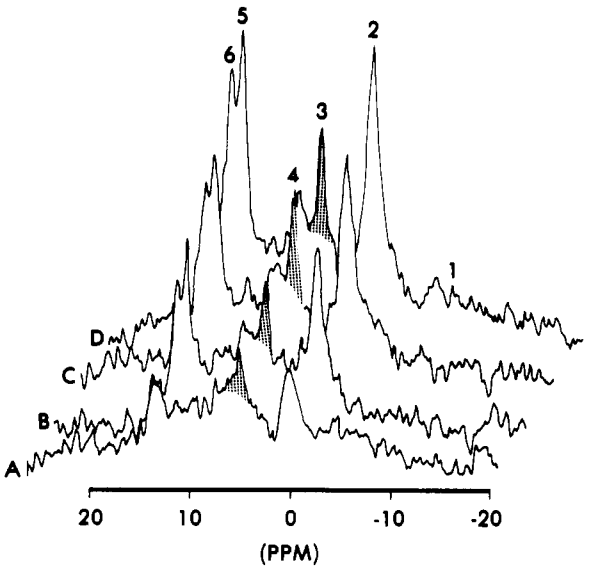

FIGURE 6: Spectra used to obtain $T_{1\,app}$ of γATP with both PCr and βATP (missing peaks 1 and 4) saturated. The area of the γATP resonance was measured at (A) 100 ms, (B) 450 ms, (C) 900 ms, and (D) 1.5 s after saturation of the whole spelctrum. The PCr and βATP peaks were kept continually saturated. Peaks are numbered as in Figure 1.

Table III: Effects of Saturating PCr and/or βATP on the γATP, βATP, and PCr Peaks and the $T_{1\,app}$ of γATP

obsd peak	M^{ss}/M^{0} saturating PCr	M^{ss}/M^{0} saturating βATP	$T_{1\,app}$ (s), saturating both PCr and βATP
γATP	0.73 (n = 7)	0.80 (n = 3)	0.63 (n = 3)
βATP	1 (n = 3)		
PCr		0.92 (n = 3)	

where $M^{ss}{}_{\gamma ATP}$ is the steady-state magnetization of γATP with PCr saturated in eq 2 and βATP saturated in eq 3, $M^{0}{}_{\gamma ATP}$ is the equilibrium magnetization of the γATP signal measured with a suitable control irradiation pulse, $M^{ss}{}_{\beta ATP}$ is the steady-state magnetization of βATP with PCr saturated, $M^{0}{}_{\beta ATP}$ is the equilibrium magnetization of βATP, $M^{ss}{}_{PCr}$ is the steady-state magnetization of PCr with βATP saturated, $M^{0}{}_{PCr}$ is the equilibrium magnetization of PCr, and $T_{1\,\gamma ATP}$ is the intrinsic T_1 of γATP. The $T_{1\,app}$ of γATP with both PCr and βATP saturated is given by

$$T_{1\,app} = \frac{T_{1\,\gamma ATP}}{1 + (k_{ATP\rightarrow PCr} + k_{\gamma ATP\rightarrow\beta ATP})T_{1\,\gamma ATP}} \quad (4)$$

Thus, measurement of $M^{ss}{}_{\gamma ATP}/M^{0}{}_{\gamma ATP}$ and $M^{ss}{}_{\beta ATP}/M^{0}{}_{\beta ATP}$ with the PCr resonance saturated, $M^{ss}{}_{\gamma ATP}/M^{0}{}_{\gamma ATP}$ and $M^{ss}{}_{PCr}/M^{0}{}_{PCr}$ with the βATP resonance saturated, and $T_{1\,app}$ of γATP with both the PCr and βATP signals saturated (Figure 6; a third rf synthesizer was used for this purpose) allows the rate constant $k_{ATP\rightarrow PCr}$ to be determined. A similar approach has been used previously to measure proton exchange (Perrin & Johnston, 1979).

Table III presents values for the different parameters measured in three different hearts. The rate constant derived from these measurements is 0.44 ± 0.08, which is not significantly different from that measured by assuming a two-site exchange between PCr and γATP. These results suggest that the effect of exchange between β- and γATP was not sufficient to explain the discrepancy in the measured forward and reverse rates of the creatine kinase reaction in this system.

Discussion

Kidney. Recent results in the perfused kidney (Freeman et al., 1983) interpreted the measured exchange between P_i and γATP to be due to the net synthesis of ATP via oxidative phosphorylation. If whole kidney ATP concentration is assumed to be 1.7 μmols/g wet weight (Hems & Brosnan, 1970), and the P_i/ATP ratio is 1.03 (present results), then a value of 1.8 μmol/g wet weight for whole kidney P_i concentration can be derived. Multiplying this P_i concentration by the measured rate constant (Table I) gives a rate of 0.22 μmol s^{-1} (g wet weight)$^{-1}$ of 13 μmol min^{-1} (g wet weight)$^{-1}$ for the incorporation of P_i into ATP. Previously published rates of oxygen consumption and sodium reabsorption for the intact rat kidney range from 4 to 9 μmol of O_2 min^{-1} (g wet weight)$^{-1}$ and 48–128 μmol of Na^+ min^{-1} (g wet weight)$^{-1}$ (Weinstein & Szyjewicz, 1974). Using these values, it is estimated that the flux ratio J_{ATP}/J_O is in the range of 0.8–1.7 and the J_{Na^+}/J_{ATP} ratio ranges from 4 to 10. While the J_{ATP}/J_O value is low and the J_{Na^+}/J_{ATP} ratio is higher than generally accepted, these estimates are consistent with the hypothesis that saturation transfer measures net ATP synthesis in a physiologically relevant range. Experiments to simultaneously measure NMR parameters, O_2 consumption, and Na^+ reabsorption in the same animal are in progress.

There are several problems concerning the general interpretation of saturation transfer experiments in vivo. Several of these problems complicate interpretation of the kidney results. First, as much as 30% of the P_i peak intensity could be due to extracellular P_i plus the 2-phosphate of 2,3-diphoshoglycerate (2,3-DPG) (Saki et al., 1974). However, two recent reports from the same group quantitated the amount of P_i in phosphate-free perfused kidney (Freeman et al., 1983) and kidney in situ (Stubbs et al., 1984) and found the same value. This indicates that extracellular P_i and 2,3-DPG make an insignificant contribution to the P_i peak. Nevertheless, if extracellular P_i and 2,3-DPG did contribute to the P_i peak, this would lead to an underestimate of $(M^0 - M^{ss})/M^0$ for P_i. A significant nonexchanging pool would, however, also lead to an overestimate of the exchanging P_i concentration, which would balance the error in $(M^0 - M^{ss})/M^0$ in the calculation of the rate of P_i incorporation in ATP. However, the $T_{1\,app}$ of the P_i peak will also be affected by extracellular P_i and 2,3-DPG. The 2-phosphate of 2,3-DPG in arterial blood has a T_1 of approximately 2.2 s at 97.2 MHz. Extracellular P_i would also be expected to have a longer T_1 than intracellular P_i. These influences tend to cause overestimation of the $T_{1\,app}$ of intracellular P_i. If 2,3-DPG contributes 20% to the P_i peak, then from the measured $T_{1\,app}$ of 1.24 s for the P_i peak, a value of 1 s can be derived for the $T_{1\,app}$ of intracellular P_i. This implies that the rate constant and the rate of ATP synthesis were underestimated, possibly explaining the low J_{ATP}/J_O and high J_{Na^+}/J_{ATP} ratios obtained. The low signal to noise ratio of the P_i peak makes separating the various components of the P_i relaxation curve with a multiexponential fit difficult. This might be possible if more time were used to make the T_1 measurement. Another alternative would be to perform the experiment by varying the time that the γATP resonance is saturated (Forsén & Hoffman, 1963; Nunnally & Hollis, 1979). In this case, the time dependence of the decay of P_i magnetization would be due only to the exchanging component of the peak. To be reliable, this technique requires a greater signal to noise ratio than obtained in these experiments.

A second problem of interpretation is whether the rate of P_i–ATP exchange represents net ATP synthesis. In experiments with perfused heart (Matthews et al., 1981), kidney (Freeman et al., 1983), and maize root tips (Roberts et al., 1984), an expected J_{ATP}/J_O ratio of approximately 3 was obtained. However, in yeast, the J_{ATP}/J_O ratio was found to be 88 (Alger et al., 1982), suggesting that a rapid $P_i \rightleftharpoons$ ATP exchange rather than net synthesis was being measured. It is possible that a component of the $P_i \rightleftharpoons$ ATP exchange measured in kidney does not represent net ATP synthesis. Finally, saturation transfer studies to measure ATP synthesis are based upon the assumption that all of the P_i participating in the exchange reaction is visible to NMR. Freeman et al. (1983) reported that only 25% of the total P_i was visible to NMR in the perfused kidney. It is possible that the rate of ATP synthesis is overestimated because of rapid P_i–ATP exchange and underestimated because of NMR-invisible metabolites. Further experiments are necessary to examine these possibilities.

Heart. PCr is generally considered to be a reservoir of high-energy phosphate for times when work demands on a tissue rapidly increase (Lehninger, 1975). To satisfy this role, the rate of the creatine kinase reaction must be significantly larger than the rate of ATP synthesis or utilization. In order to compare our measured rates of creatine kinase activity to ATP synthesis rates, it is assumed that values for oxygen consumption and ATP concentrations in the perfused working heart approximate those found in vivo. With an oxygen consumption rate of 1.4 μmol s^{-1} (g dry weight)$^{-1}$ (Taegtmeyer et al., 1980), an ATP synthesis rate of 8 μmol s^{-1} (g dry weight)$^{-1}$ [3.5 mM/s, making corrections for cytoplasmic volume (Matthews et al., 1982)] can be calculated by assuming J_{ATP}/J_O equals 3. An assumed ATP concentration of 33 μmol/g dry weight (Wikman-Coffelt et al., 1983) with our NMR-determined values of [PCr]/[ATP] allows the forward and reverse rates of creatine kinase to be estimated as 24 μmol s^{-1} (g dry weight)$^{-1}$ (10 mM/s) and 17 μmol s^{-1} (g dry weight)$^{-1}$ (7 mM/s), respectively. Thus, the forward rate through creatine kinase is approximately 3 times higher than the rate of ATP synthesis. This agrees with results obtained from the isolated perfused rat heart (Matthews et al., 1982), where the forward rate through creatine kinase ranged from 13 to 3.3 times the ATP synthesis rate as the workload increased toward that found in situ. The value of 24 μmol s^{-1} (g dry weight)$^{-1}$ (10 mM/s) estimated for the forward rate of creatine kinase in situ is somewhat larger than that found for the Langendorff-perfused heart at high workloads (4.6 mM/s; Matthews et al., 1982) and smaller than the value of 35 μmol s^{-1} (g dry weight)$^{-1}$ reported for the pyruvate-perfused working heart (Kobayashi et al., 1982).

The present experiments measured a 33% difference in the forward and reverse rates of the creatine kinase reaction for the heart in situ. This discrepancy in rates has also been measured in the perfused heart (Brown et al., 1979; Nunnally & Hollis, 1979; Matthews et al., 1982; Kobayashi et al., 1982), isolated muscle (Gadian et al., 1981), and brain in situ (Shoubridge et al., 1982). Experiments using a double saturation technique to account for a major competing exchange (i.e., between β- and γATP) demonstrate that the measured reverse rate of creatine kinase is not significantly affected by this reaction. This is in contrast to other workers who estimated that exchange catalyzed by ATP synthesis and adenylate kinase could account for the measured discrepancy (Matthew et al., 1982). However, these calculations only accounted for the effects of the competing reaction on M^0 –

$M^{ss})/M^0$ of γATP and assumed that the effects on $T_{1\,app}$ were negligible. The double saturation technique utilized in the present study accounts for the effect of competing reactions on both $T_{1\,app}$ and $(M^0 - M^{ss})/M^0$.

Recently, Ugurbil reported that in the pyruvate-perfused heart, exchange between P_i and γATP did explain the discrepancy in rates measured for the creatine kinase reaction (Ugurbil, 1984). To eliminate the effects of $P_i \rightleftharpoons \gamma$ATP exchange, Ugurbil measured the reverse rate of the creatine kinase reaction while maintaining P_i saturated. In the heart in situ, preliminary experiments presented under Results suggested that there was no significant difference between the reverse rate measured with or without P_i saturated. The reasons for this discrepancy between the perfused and in situ heart are not clear. One difficulty in performing this experiment in the heart in situ is that the intracellular P_i resonance is masked by a large signal from 2,3-DPG in blood. In addition, measurement error may have prevented detection of small changes. Ideally, the saturation transfer experiment should be performed by saturating all potential sites which may be in exchange with γATP while measuring the γATP intensity and $T_{1\,app}$.

Another explanation offered for the difference in forward and reverse rates of creatine kinase invokes the notion of intracellular compartmentation (Nunnally & Hollis, 1979). It is interesting to note that the difference in creatine kinase rates measured in the heart in situ [7 μmol s^{-1} (g dry weight)$^{-1}$] is roughly equal to the estimate of the ATP synthesis rate [8 μmol s^{-1} (g dry weight)$^{-1}$]. This observation suggests that a model of compartmentation based on the "shuttle" hypothesis (Bessman & Geiger, 1981) may explain the discrepancy in measured creatine kinase rates (Koretsky & Weiner, 1984). The model assumes that, in addition to cytoplasmic ATP exchanging with PCr, there is a small pool of ATP, sequestered in or near the mitochondria, reacting to form PCr at a rate equal to the rate of ATP synthesis. Further experiments correlating the rate of ATP synthesis with the difference in the forward and reverse rates of the creatine kinase catalyzed reaction should help to clarify the situation.

Acknowledgments

We acknowledge support and helpful discussions with William Parmley, Floyd C. Rector, Jr., Vladimir Basus, Joseph Murphy-Boesch, and Greg Karczmar. Dr. Ai Xuan performed some of the animal surgery.

Registry No. 5′-ATP, 56-65-5; phosphate, 14265-44-2; phosphocreatine, 67-07-2; creatine kinase, 9001-15-4.

References

Ackerman, J. J. H., Evelhoch, J. L., Berkowitz, B. A., Kichura, G. M., Deuel, R. K., & Lown, K. S. (1984) *J. Magn. Reson. 56*, 318–322.

Alger, J. R., den Hollander, J. A., & Shulman, R. G. (1982) *Biochemistry 21*, 2957–2963.

Balaban, R. S., Kantor, H. L., & Ferretti, J. A. (1983) *J. Biol. Chem. 258*, 12787–12789.

Bessman, S. P., & Geiger, P. J. (1981) *Science (Washington, D.C.) 211*, 448–452.

Brown, T. R., Ugurbil, K., & Shulman, R. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5551–5553.

Brown, T. R., Gadian, D. G., Garlick, P. B., Radda, G. K., Seeley, P. J., & Styles, P. (1979) in *Frontiers of Biological Energetics* (Dutton, P. L., Leigh, J. S., & Scarpa, A., Eds.) Vol. 2, pp 1341–1349, Academic Press, New York.

Forsén, S., & Hoffman, R. A. (1963) *J. Chem. Phys. 39*, 2892–2901.

Freeman, D., Bartlett, S., Radda, G., & Ross, B. (1983) *Biochim. Biophys. Acta 762*, 325–336.

Gadian, D. G., Radda, G. K., Brown, T. R., Chance, E. M., Dawson, J. S., & Wilkie, D. R. (1981) *Biochem. J. 194*, 215–228.

Gonzalez-Mendez, R., Litt, L., Koretsky, A. P., von Colditz, J., Weiner, M. W., & James, T. L. (1984) *J. Magn. Reson. 57*, 526–533.

Gupta, R. K. (1979) *Biochim. Biophys. Acta 586*, 189–195.

Hansford, R. G. (1980) *Curr. Top. Bioenerg. 10*, 217–278.

Hems, P. A., & Brosnan, J. T. (1970) *Biochem. J. 120*, 105–111.

Kobayashi, K., Fossel, E., & Ingwall, J. (1982) *Biophys. J. 37*, 123a.

Koretsky, A. P., & Weiner, M. W. (1984) in *Biomedical Magnetic Resonance* (James, T. L., & Margulis, A. R., Eds.) pp 209–230, Radiology Research and Education Foundation, San Francisco, CA.

Koretsky, A. P., Strauss, W., Basus, V., Murphy, J., Bendel, P., James, T. L., & Weiner, M. W. (1982) in *Acute Renal Failure* (Eliahu, H. E., Ed.) pp 42–46, John Libbey, London.

Koretsky, A. P., Wang, S., Klein, M. P., James, T. L., & Weiner, M. W. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7491–7496.

Koretsky, A. P., Wang, S., Klein, M. P., James, T. L., & Weiner, M. W. (1984) *Magn. Reson. Med. 1*, 186–187.

Lehninger, A. L. (1975) in *Biochemistry*, 2nd ed., p 406, Worth, New York.

Mandel, L. J., & Balaban, R. S. (1981) *Am. J. Physiol. 240*, F357–F371.

Matson, G. B., Schleich, T., Serdahl, C., Acosta, G., & Willis, J. A. (1984) *J. Magn. Reson. 56*, 200–206.

Matthews, P. M., Bland, J. L., Gadian, D. G., & Radda, G. K. (1981) *Biochem. Biophys. Res. Commun. 103*, 1052–1059.

Matthews, P. M., Bland, J. L., Gadian, D. G., & Radda, G. K. (1982) *Biochim. Biophys. Acta 721*, 312–320.

McDonald, G. G., & Leigh, J. S. (1973) *J. Magn. Reson. 9*, 358–370.

Murphy-Boesch, J., & Koretsky, A. P. (1983) *J. Magn. Reson. 54*, 526–532.

Nunnally, R. L., & Hollis, D. P. (1979) *Biochemistry 18*, 3642–3646.

Perrin, C. L., & Johnston, E. R. (1979) *J. Magn. Reson. 33*, 619–626.

Roberts, J. K. M., Wemmer, D., & Jardetzky, O. (1984) *Plant Physiol. 74*, 632–639.

Saki, R., Ikura, K., Sugimoto, E., & Chiba, H. (1974) *Anal. Biochem. 61*, 43–47.

Shoubridge, E. A., Briggs, R. W., & Radda, G. K. (1982) *FEBS Lett. 140*, 288–292.

Stubbs, M., Freeman, D., & Ross, B. D. (1984) *Biochem. J. 224*, 241–246.

Taegtmeyer, H., Hems, R., & Krebs, H. A. (1980) *Biochem. J. 186*, 701–711.

Ugurbil, K. (1984) Abstracts of the International Conference on Magnetic Resonance in Biological Systems, XI, Sept 17–23, Goa, India.

Weiner, M. W., Green, K., Vremean, H., Wemmer, D., Wade-Jardetzky, N., & Jardetzky, O. (1980) *IRCS Med. Sci. 8*, 671.

Weinstein, S. W., & Szyjewics, J. (1971) *Am. J. Physiol. 227*, 171-177.
Wemmer, D., Wade-Jardetzky, N., Robin, E., & Jardetzky, O. (1982) *Biochim. Biophys. Acta 720*, 281-287.
Wikman-Coffelt, J., Sievers, R., Coffelt, R. S., & Parmley, W. W. (1983) *Am. J. Physiol. 245*, H354-H362.
Yahaya, H., Chan, L., Freeman, D., Holloway, P., & Ross, B. (1984) *Clin. Sci. 66*, 35p.

Metal Ion Binding Properties of Hen Ovalbumin and S-Ovalbumin: Characterization of the Metal Ion Binding Site by ^{31}P NMR and Water Proton Relaxation Rate Enhancements†

Warren J. Goux* and P. N. Venkatasubramanian
Department of Chemistry, The University of Texas at Dallas, Richardson, Texas 75083-0688
Received May 22, 1985

ABSTRACT: In this study, water proton relaxation rate (PRR) enhancements have been used to characterize the binding of metal ions to native ovalbumin, ovalbumin in which phosphate has been enzymatically cleaved from one or both of the two protein phosphoserines, and a heat-stabilized form of the protein (S-ovalbumin). With Scatchard plots constructed from water PRR enhancements, it was found that native ovalbumin and S-ovalbumin had one strong binding site for Mn^{2+} ion ($K_D \simeq 6.0 \times 10^{-4}$ M). Alkaline phosphatase treated ovalbumin, a protein having a single phosphoserine, had one Mn^{2+} binding site of slightly weaker affinity ($K_D \simeq 8.3 \times 10^{-4}$ M), while acid phosphatase treated ovalbumin, a dephosphorylated protein, had two much weaker Mn^{2+} ion binding sites ($K_D \simeq 1.3 \times 10^{-3}$ M). Competitive binding studies on the native protein suggested that Zn^{2+} ion competes with Mn^{2+} for the single strong-affinity site ($K_D \simeq 6.1 \times 10^{-3}$ M) while Mg^{2+} and Ca^{2+} do not. In a second set of experiments, the paramagnetic contribution to the ^{31}P spin-lattice (T_{1P}) and spin-spin (T_{2P}) relaxation times at three separate magnetic field strengths was measured. Correlation times τ_c characterizing Mn^{2+}-^{31}P dipolar relaxation were estimated from the ratios of T_{1P}/T_{2P} at a single field and from the ratios of spin-lattice relaxation rates at three different field strengths. The correlation times so obtained, ranging from about 0.7 to 7.7 ns at the three field strengths, were used in calculating distances from the bound Mn^{2+} ion to the phosphoserines of native ovalbumin, S-ovalbumin, and alkaline phosphatase treated ovalbumins. It was determined that the phosphate of phosphoserine-68 was 5.95 ± 0.26 and 6.29 ± 0.18 Å from the Mn^{2+} in the native and alkaline phosphatase treated protein, respectively, and 6.99 ± 0.30 Å away from the Mn^{2+} in S-ovalbumin. The phosphate of phosphoserine-344 was determined to be 5.31 ± 0.20 and 5.75 ± 0.10 Å from the Mn^{2+} ion in native ovalbumin and S-ovalbumin, respectively. The ^{13}C nucleus of [1-^{13}C]galactose enzymatically transferred to the nonreducing end of the ovalbumin oligosaccharide chain was not found to be significantly relaxed by Mn^{2+} bound to the protein, even at 1:1 stoichiometric ratio of metal:protein. Using this, we estimate the nonreducing terminal of the ovalbumin oligosaccharide to be at least 39 Å from the metal ion binding site on the protein.

Hen ovalbumin is a soluble glycoprotein (M_r 42 700) having a single carbohydrate chain covalently linked to the nitrogen amide of an asparagine residue (Taborsky, 1974; Nisbet et al., 1981). Although the tertiary structure has not been determined by X-ray crystallography, the complete primary sequence of its 348 amino acid residues is known (Nisbet et al., 1981; McReynolds et al., 1978). The protein has been shown to be heterogeneous, both with respect to its attached carbohydrate chain (Atkinson et al., 1981; Tai et al., 1975; Narashiman et al., 1980) and with respect to the degree of phosphorylation of two of its serine side chains (Linderstrom-Lang & Ottesen, 1949). Heterogeneity of the protein due to varying degrees of phosphorylation may be removed by ion-exchange chromatography (Rhodes et al., 1958). The major protein component has two phosphoserines present, while minor components bear only one of the two phosphoserines per protein equivalent. When the native protein is warmed under alkaline conditions, a conformational change takes place and native ovalbumin is converted to a more heat-resistant form (S-ovalbumin) (Smith & Black, 1968a,b; Donovan & Mapes, 1976). The S form of the protein is hydrodynamically more compact and has a greater number of tritratable groups exposed to the protein surface (Nakamura et al., 1981, 1980; Nakamura & Masuko, 1981; Kint & Tomimatsu, 1979). Although the functional significance of ovalbumin is not known, it has for some time been thought responsible for metal ion transport and storage (Taborsky, 1974). However, only the Mg^{2+} binding characteristics of the protein have been studied (Vogel & Bridger, 1982).

In this study, water proton relaxation rate (PRR) enhancements and ^{31}P NMR relaxation rate data are used to determine the metal ion binding properties, titratability of the two phosphoserines, and distances from protein-bound paramagnetic Mn^{2+} to each of the two phosphoserines of native ovalbumin and its conformational variant, S-ovalbumin. In an attempt to determine changes in Mn^{2+} ion-carbohydrate

† This work was supported by Grant AT-885 from the Robert A. Welch Foundation and by BRSG S07 RR07133 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health.
* Author to whom correspondence should be addressed.

0006-2960/86/0425-0084$01.50/0