tellano, P. R., Ed.) pp 119–160, Plenum Press, New York.
Kawato, S., & Kinosita, K., Jr. (1981) *Biophys. J. 36*, 277–296.
Kawato, S., Kinosita, K., Jr., & Ikegami, A. (1977) *Biochemistry 16*, 2319–2324.
Kawato, S., Sigel, E., Carafoli, E., & Cherry, R. J. (1980) *J. Biol. Chem. 255*, 5508–5510.
Kawato, S., Sigel, E., Carafoli, E., & Cherry, R. J. (1981) *J. Biol. Chem. 256*, 7518–7527.
Kawato, S., Gut, J., Cherry, R. J., Winterhalter, K. H., & Richter, C. (1982a) *J. Biol. Chem. 257*, 7023–7029.
Kawato, S., Lehner, C., Mueller, M., & Cherry, R. J. (1982b) *J. Biol. Chem. 257*, 6470–6476.
Kawato, S., Mitani, F., Iizuka, T., & Ishimura, Y. (1988) *J. Biochem. 104*, 188–191.
Kawato, S., Ashikawa, I., Iwase, T., & Hara, E. (1991) *J. Biochem. 109*, 587–593.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951), *J. Biol. Chem. 193*, 265–275.
Mueller, M., Krebs, J. J. R., Cherry, R. J., & Kawato, S. (1984) *J. Biol. Chem. 259*, 3037–3043.
Murakami, H., Yabusaki, Y., Sakaki, T., Shibata, M., & Ohkawa, H. (1987) *DNA 6*, 189–197.
Murakami, H., Yabusaki, Y., Sakaki, T., Shibata, M., & Ohkawa, H. (1990) *J. Biochem. 108*, 859–865.
Oeda, K., Sakaki, T., & Ohkawa, H. (1985) *DNA 4*, 204–210.
Ohta, Y., Mitani, F., Ishimura, Y., Yanagibashi, K., Kawamura, M., & Kawato, S. (1990) *J. Biochem. 107*, 97–104.
Ohta, Y., Yanagibashi, K., Hara, T., Kawamura, M., & Kawato, S. (1991) *J. Biochem. 109*, 594–599.
Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370–2378.
Sakaki, T., Oeda, K., Miyoshi, M., & Ohkawa, H. (1985) *J. Biochem. 98*, 167–175.
Sakaki, T., Shibata, M., Yabusaki, Y., & Ohkawa, H. (1987) *DNA 6*, 31–39.
White, R. J., & Coon, M. J. (1980) *Annu. Rev. Biochem. 49*, 315–356.
Yabusaki, Y., Murakami, H., Sakaki, T., Shibata, M., & Ohkawa, H. (1988) *DNA 7*, 701–711.

^{31}P NMR Saturation-Transfer Study of the in Situ Kinetics of the Mitochondrial Adenine Nucleotide Translocase†

Peter T. Masiakos,‡ Gerald D. Williams,§ Deborah A. Berkich,‡ Michael B. Smith,‡,§,∥ and Kathryn F. LaNoue*,‡

Departments of Cellular and Molecular Physiology, Radiology, Division of NMR Research, and Biological Chemistry, The Milton S. Hershey Medical Center, College of Medicine, The Pennsylvania State University, Hershey, Pennsylvania 17033

Received October 22, 1990; Revised Manuscript Received April 1, 1991

ABSTRACT: The exchange of intramitochondrial ATP (ATP_{in}) for extramitochondrial ATP (ATP_{out}) was measured by using ^{31}P NMR spectroscopy over a range of temperatures in isolated rat liver mitochondria oxidizing glutamate and succinate in the presence of external ATP but no added ADP (state 4). The rate of this exchange is more than an order of magnitude faster than rates reported previously that were determined by using isotopic techniques in the presence of oligomycin, the potent ATPase inhibitor. Differences are ascribed in part to the low levels of matrix ATP present in oligomycin-treated mitochondria. The addition of oligomycin to mitochondrial suspensions decreases intramitochondrial ATP levels from 17 ± 3 (SEM) nmol/mg of protein in state 4 to 1.51 ± 0.1 nmol/mg of protein in the presence of inhibitor at 8 °C. Simultaneously, transporter flux falls from 960 ± 55 nmol/min·mg to undetectable levels (less than 300 nmol/min·mg). Although transport rates are much faster when measured by saturation-transfer than by conventional isotopic methods, the enthalpy values obtained by determining the effect of temperature on flux are very similar to those reported in the past that were determined by using isotopic techniques. Intramitochondrial ATP content regulates the rate of the ATP_{in}/ATP_{out} exchange. At 18 °C, the concentration of internal ATP that produces half-maximal transport rate is 6.6 ± 0.12 nmol/mg of mitochondrial protein. The relationship between substrate concentration and flux is sigmoidal and is 90% saturated at 11.3 ± 0.18 nmol/mg of mitochondrial protein. Since the measured rates of exchange of ATP_{in} for ATP_{out} are almost 10 times faster than the ATP synthase (ATP/P_i) exchange rates, the translocase cannot limit net ATP/P_i exchange in state 4. It may, nonetheless, limit net synthesis of ATP under other conditions when matrix ATP concentration is lower than in state 4 and when external ADP is present at higher concentrations than in these experiments.

Chance and Williams (1956) proposed that mitochondrial respiration is tightly controlled by ADP availability. The site of control by ADP remains uncertain. The adenine nucleotide translocase, the electrogenic mitochondrial membrane transporter that catalyzes the exchange of nucleotides, has been suggested as a rate-controlling step for net ATP synthesis (Heldt, 1966, 1967; Heldt & Klingenberg, 1968; Kemp et al., 1969). Previous work aimed at identifying the important determinants of mitochondrial respiration has generated two main hypotheses. The first is the "near-equilibrium hypothesis" of Ericinska and Wilson (1982), which suggests that the electron transport chain and the cytosolic phosphorylation

† This work was supported by NIH Grant P01 HL18708 (K.F.L.).
* Corresponding author.
‡ Department of Cellular and Molecular Physiology.
§ Department of Radiology.
∥ Department of Biological Chemistry.

0006-2960/91/0430-8351$02.50/0

potential are in near equilibrium. Thus, the rate of O_2 consumption is set by the redox state of cytochrome aa_3, which is a function of the external phosphorylation potential, $(ATP)/[(ADP)(P_i)]$. Reasoning that the adenine nucleotide translocase is an intermediate step in this reaction sequence, they concluded that it cannot be rate limiting. Still others have shown that the extramitochondrial ATP/ADP ratio and not the external phosphorylation potential influences the activity of enzymes involved in ATP synthesis (Davis & Davis-van Thienen, 1978; Kunz et al., 1981; Burat et al., 1984; Verhoeven et al., 1985). This control may occur at the level of the adenine nucleotide translocase, which influences net ATP synthesis by limiting the transport of ADP across the mitochondrial inner membrane. LaNoue and co-workers (1986) have recently shown that the ATP synthase is usually not in equilibrium by demonstrating that the forward and back reactions under physiological conditions are not rapid with respect to the net flux. The forward reaction of ATP synthesis measured isotopically as formation of radiolabeled ATP from radiolabeled P_i is not dramatically changed by ADP availability, while the reversal of the synthase reaction (hydrolysis of ATP) is decreased dramatically by increased ADP availability and is therefore an important determinant of net flux. It is possible that ADP, by competing with ATP at the level of the translocase, limits entry of ATP into the mitochondria and thereby restricts the backward reaction. Studies designed to identify the causes of this modulation of the back reaction and the site of modulation are important because they may define determinants of in situ ATP synthesis.

The mitochondrial adenine nucleotide translocase has been extensively investigated in a wide variety of cells and by many different techniques [reviewed by LaNoue and Schoolwerth (1984), Klingenberg (1976), and Vignais (1976)]. Although these studies have generated a significant amount of information regarding its structure and function, little is known about its kinetics in coupled mitochondria. Much of the available kinetic information on the translocase has been obtained from studies using isotopically labeled substrates (Barbour & Chan, 1981; Duyckaerts et al., 1980). These classical biochemical methods are technically inadequate due to their inherently poor temporal resolution and the rapidity with which the small mitochondrial nucleotide compartment equilibrates with the large external compartment. Reliable initial rates are therefore very difficult to measure.

The study of the kinetics of enzymes in intact cells and tissues has been greatly facilitated by the development of nuclear magnetic resonance (NMR) saturation-transfer techniques [reviewed by Alger and Shulman (1984), Koretsky and Weiner (1984), Brindle and Campbell (1987), Ugurbil (1985), and Kuchel (1990)]. NMR saturation-transfer methodology has also been used successfully to study the kinetics of individual enzymes that are involved in intimately coupled biochemical processes [Brown et al. (1977), Zweier and Jacobus (1987), Bittl et al. (1987), Campbell-Burk et al. (1987), Degani et al. (1987), Hsieh and Balaban (1988), Rees et al. (1989), and Brindle et al. (1989) to name a few representative ^{31}P examples]. Therefore, NMR saturation-transfer affords a means by which activities of specific enzymes with rapid kinetics could be assessed noninvasively, in situ.

Previous studies from our laboratories (Hutson et al., 1989) have shown that it is possible to measure internal and external ATP by nuclear magnetic resonance spectroscopy in respiring mitochondria. In the absence of added ADP but in the presence of a respiratory subtrate, well-coupled liver mitochondria use O_2 very slowly. External ATP/ADP ratios were maintained at high levels and respiratory control was preserved in the NMR spectrometer for over an hour at both 8 and 25 °C by using O_2 bubbling techniques. The availability of this technology now permits measurements of adenine nucleotide transport to be made by NMR saturation-transfer techniques in actively respiring mitochondria with active ATP/P_i exchange properties.

Materials and Methods

Mitochondrial Isolation. Mitochondria were isolated from male Sprague–Dawley rats (250–500 g). After administration of pentobarbital (150 mg/kg) as anesthetic (IP), a ventral midline incision was made. The exposed livers were perfused with approximately 200 mL of an ice-cold solution of 225 mM mannitol, 75 mM sucrose, 5 mM MOPS,[1] 1 mM EGTA, and 0.1 mM EDTA (MSEE) at pH 7.0. The livers were excised, minced, rinsed, and homogenized with MSEE. Mitochondrial isolation was then carried out by standard techniques of differential centrifugation (Schneider & Hogeboom, 1950). NMR experiments were performed on mitochondria resuspended in a solution containing 130 mM KCl, 15 mM succinate, 15 mM glutamate, 20 mM MOPS, and 1 mM CDTA, pH 6.8. The mitochondrial protein and external ATP were adjusted for each temperature to maximize the observable transfer of magnetization. An adequate supply of O_2 was maintained by continuously bubbling the suspension with 100% oxygen during signal acquisition. At the conclusion of each experiment, samples were taken for the determination of intramitochondrial and extramitochondrial adenine nucleotide content, as described previously (Hutson et al., 1989).

Loading Mitochondria with ATP. Mitochondria (4 mg/mL) were loaded with ATP at 30 °C, by a variation of the method described Austin and Aprille (1984). The ATP-loaded mitochondria exhibit only minimal loss of ATP to the medium following loading when extramitochondrial Ca^{2+} and P_i are maintained at low levels (Aprille, 1988).

Assays. Mitochondrial protein was determined by the biuret method in the presence of 0.125% deoxycholate. Determination of mitochondrial oxygen consumption was carried out at 37 °C with the mitochondria suspended in a solution containing 150 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 20 mM MOPS, and 0.05 mM EGTA at pH 7.2. ADP-stimulated (state 3) respiration was initiated by the addition of 0.5 mM ADP to mitochondria that contained 20 mM glutamate and 1 mM malate. Respiratory control ratios (RCR) or the ratio of O_2 consumption in the presence of excess ADP and phosphate (state 3) compared to O_2 consumption in the absence of ADP (state 4) exceeded 6 for all reported experiments. Oxygen consumption was measured polarographically with a Clark electrode. Adenine nucleotides were determined spectrophotometrically (Williamson & Corkey, 1969).

NMR Measurements. ^{31}P NMR spectra were acquired at 162.0 MHz with a Bruker AM-400 wide-bore spectrometer equipped with a 20-mm $^{13}C/^{31}P$ double-tuned probe. An 8-mL mitochondrial sample (8–40 mg/mL) was placed in a 15-mm NMR tube that was inserted into a 20-mm NMR tube containing ^2H_2O. The magnetic field homogeneity was optimized by using the water proton signal of the sample while maintaining a deuterium frequency lock. The Mg^{2+} chelator CDTA

[1] Abbreviations: CDTA, (*trans*-1,2-diaminocyclohexane)-*N,N,N',N'*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; MOPS 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); T_1, spin-lattice relaxation time; β-ATP, β-phosphorus nuclei of ATP, observable in ^{31}P NMR spectra of ATP.

was used to separate the external from internal adenine nucleotide species in the NMR spectrum. Since the visibility of the intramitochondrial ATP was compromised by the long delays required and the poor signal-to-noise ratio of the saturation-transfer experiments, reference spectra were recorded before each saturation-transfer experiment using a 68° flip angle and a relaxation delay of 0.3 s in order to measure the internal ATP resonance frequency.

In previous studies from this laboratory, it has been shown that when mitochondria are suspended in a Mg^{2+}-free buffer after various degrees of ATP loading, there is a 1:1 correlation of total biochemically measured matrix ATP with the NMR spectral peak that corresponds to the fully Mg^{2+} bound β-phosphate peak of ATP. This suggests that there is a single pool of metabolically active intramitochondrial ATP that can be fully saturated.

Saturation-Transfer Experiments. Magnetization-transfer measurements were made using a slight variation of the method described previously (Gadian et al., 1981). Selective radio frequency (rf) "saturation" of internal β-ATP was obtained by using a DANTE pulse sequence (Morris & Freeman, 1978) consisting of a 3.34-s train of 13 300 individual 1.1-μs pulses, each separated by a 250-μs delay. The steady-state saturation was briefly interrupted by a nonselective 26-μs (90°) pulse and a signal acquisition time of 86 ms. The saturating power level did not increase temperature by more than 1 °C under the conditions of the experiment. The corrected temperature was used for all calculations and figures. Each spectrum was obtained from the sum of three blocks of 50 transients each and was compared with a similarly obtained control spectrum. The acquisition time for the control was identical, but the DANTE pulse was applied at a frequency upfield to the external peak by a distance equal to the separation between the internal and external signals of β-ATP. Since the two spectra were averaged over three blocks, time-dependent effects are greatly reduced. Since the separation between the internal and external β-ATP resonances was close to 500 Hz, the saturating rf pulse was localized to the resonance of interest with any minimal rf error corrected for by the control condition (Kuhn et al., 1986). A progressive-saturation experiment was incorporated into the DANTE sequence for the determination of relaxation times of external β-ATP in the presence of rf saturation of internal β-ATP as shown in Figure 1. Three variable delays were chosen to optimize experimentation time and T_1's were determined from three pairs of intensity ratios according to the two-point method as described by Freeman (1972). Separate control studies under identical conditions verified that better than 98% saturation was obtained for the standard experiment and 93% saturation for the shortest delay of the T_1 experiment.

The enzymatic first-order rate constant for transport into the mitochondria was obtained by using the equation of Forsen and Hoffman (1963):

$$k_1 = (T_{1m}^{-1})(1 - M^+/M^\circ) \quad (1)$$

where M° is the equilibrium magnetization of external ATP determined from the control spectrum, M^+ is the magnetization of external ATP during saturation of internal ATP, and T_{1m} is the spin-lattice relaxation time of the external ATP measured in the presence of exchange but with saturation of the internal ATP resonance.

Results

In order to measure translocase flux of isolated respiring liver mitochondria, constant bubbling of the suspension with 100% oxygen throughout the course of the experiment proved necessary. If the rate of bubbling at different mitochondrial protein concentrations (8–40 mg/mL) was manipulated, the mitochondria remained viable and transport rates constant for at least a half hour, even at 25 °C. A small loss of spectral resolution was observed at the high bubbling rate. If at any time O_2 delivery was inadequate, it was immediately obvious from the disappearance of the external ATP. It was not necessary to add oxygen-binding fluorocarbons to the incubation medium as described previously (Hutson et al., 1989), since lower protein concentrations were used at higher temperatures. Mitochondrial integrity was assessed by observing adenine nucleotide levels during the NMR experiments and by measuring total adenine nucleotides spectrophotometrically at the beginning and end of each NMR run (see Materials and Methods).

Calculations of rates for the exchange of ATP_{in} for ATP_{out} were made from spectra similar to those shown in Figure 1. The inset of Figure 1 shows a typical reference spectrum recorded prior to each of the saturation-transfer experiments

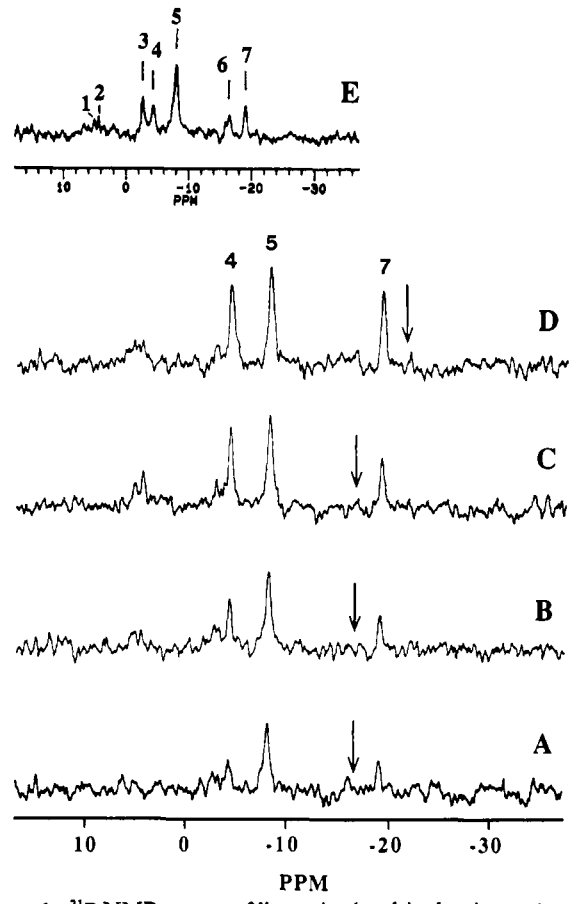

FIGURE 1: ^{31}P NMR spectra of liver mitochondria showing progressive saturation at 15 °C. The position of selective saturation is indicated by the arrows. The spectra were acquired as (A) 0.637-s delay, β-ATP_{in} irradiation; (B) 1.187-s delay, β-ATP_{in} irradiation; (C) 6.137-s delay, β-ATP_{in} irradiation; and (D) 6.137-s delay, control irradiation. Each spectrum was obtained from 150 accumulations. T_{1m} values were determined by the ratios of A:B, A:C, and B:C as described (Freeman et al., 1972; Hutson et al., 1989). Transfer of magnetization was observed from β-ATP_{out}; M^+/M° is the ratio of the areas under the β-ATP_{out} peaks in C compared to D. The concentration of external ATP was 1.4 mM. The inset (E) shows an initial reference spectrum of liver mitochondria (20 mg/mL) at 15 °C that was obtained by using 600 rapid pulses in which the resonances of intramitochondrial and extramitochondrial ATP are clearly visible. The chemical shifts of β-ATP_{in} were obtained from spectra of this type to select the correct irradiation frequency. Peak assignments are (1) $(P_i)_{in}$, (2) $(P_i)_{out}$, (3) γ-ATP_{in} + β-ADP_{in}, (4) γ-ATP_{out} + β-ADP_{out}, (5), α-ATP, α-ADP, and NAD, (6) β-ATP_{in}, and (7) β-ATP_{out}.

Table I: Effect of Temperature on ATP_{in}/ATP_{out} Exchange in Rat Liver Mitochondria[a]

temp (°C)	(*n*)	k_1 (s^{-1})	$[ATP]_{out}$ (mM)	[protein] (mg/mL)	flux (nmol/min·mg)
8	(7)	0.53 ± 0.06	1.14 ± 0.14	37.6 ± 1.5	960 ± 55
11	(3)	0.65 ± 0.12	1.07 ± 0.09	26.1 ± 1.1	1600 ± 300
15	(7)	0.63 ± 0.12	1.40 ± 0.17	25.3 ± 3.7	2100 ± 130
18	(7)	0.50 ± 0.03	1.75 ± 0.21	20.3 ± 3.0	2590 ± 80
23	(4)	0.41 ± 0.11	1.90 ± 0.40	16.4 ± 1.5	2900 ± 300
25	(1)	0.52	1.0	8.5	3670

[a] The fluxes were calculated from steady-state saturation transfer experiments described in the text. The values shown for k_1 were obtained from eq 1 (Methods), with the mean value of T_{1m} for external β-ATP taken to be 0.88 s, a value determined in separate experiments. Errors are SEM.

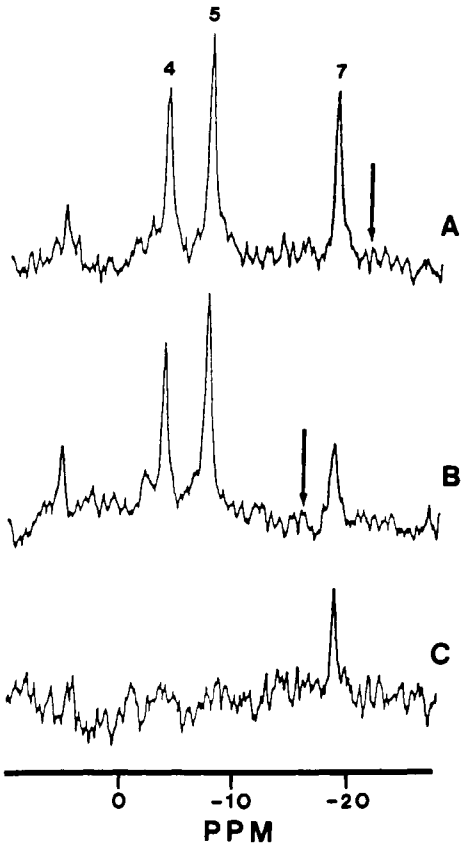

FIGURE 2: ^{31}P NMR spectra of mitochondrial adenine nucleotides showing saturation-transfer at 15 °C. The position of selective irradiation is indicated by the arrows. Each spectrum was obtained from the sum of three interleaved sets of 50 transients each, with 90° pulses and a delay of 3.34 s during which the DANTE saturation pulse train was applied. The external ATP concentration was 1.4 mM. The control spectrum is shown as spectrum A, where the DANTE pulse was applied at a frequency upfield from the β-ATP_{out} peak by a frequency equal to the separation between β-ATP_{out} and β-ATP_{in}. The difference spectrum (A − B) is shown as C.

using a 68° flip angle and a 0.3-s relaxation delay to maximize signal from internal ATP pools. Frequency assignments were obtained from these spectra for frequency-selective irradiation in the magnetization transfer experiments. Figure 1 demonstrates this experiment at 15 °C. The modified progressive-saturation experiment measures reaction rates and T_{1m}'s in less than 20 min, with no apparent degradation of mitochondrial integrity. The mean T_{1m} value for the extramitochondrial β-ATP at 8, 18, and 25 °C was 0.88 ± 0.12 s, a value similar to the 0.80 ± 0.06 s reported previously by Hutson et al. (1989) for extramitochondrial β-ATP at 8 °C. The observed temperature insensitivity of T_{1m} within the noise limitations of the experiment may be partly due to the cancelling effects of the decrease in the inherent spin-lattice relaxation rate in the absence of exchange and the increase in the exchange rate contribution with increasing temperature.

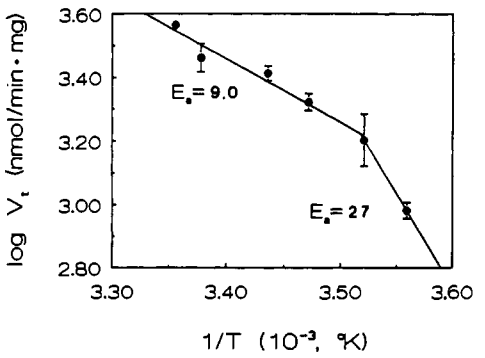

FIGURE 3: Arrhenius plot of adenine nucleotide transport versus reciprocal temperature. Temperature was varied from 8 to 25 °C. See Table I for experimental conditions.

When the ^{31}P NMR spectrum obtained by saturating the internal matrix ATP was compared with the control spectrum, the area under the media ATP was significantly decreased. The difference spectrum shown in Figure 2 and indicates that the transfer observed is significantly above the noise.

The rate of exchange of ATP_{in} for ATP_{out} was studied over a wide range of temperatures. The results, shown in Table I, confirm past observations of the strong temperature dependence of the translocase kinetics (Pfaff et al., 1969; Klingenberg, 1975). The Arrhenius plot drawn from these data (Figure 3) suggests that the activation energy (E_a) for transport is 9.0 ± 1.1 kcal/mol in the temperature range 12–25 °C. There is a break in the curve between 14 and 11 °C that increases E_a to 27 kcal/mol. These values are in good agreement with those reported for the translocase in rat liver mitochondria by Klingenberg (1976). He observed an E_a of 11 kcal/mol in the temperature range 14–37 °C, a break in the curve at 14 °C, and an E_a of 28 kcal/mol in the range 8–14 °C. However, in contrast to the good agreement between activation energies measured by the two techniques, the absolute values of transport differ by more than an order of magnitude. The absolute rates obtained in the present study using NMR saturation-transfer techniques are much higher than the isotopically generated values.

The rates measured isotopically were obtained in the presence of an inhibitor of ATP synthesis, oligomycin, whereas the present study was carried out without inhibitors. To determine the influence of oligomycin on the measurements, mitochondria were incubated at 8 °C as described in Table I, but with and without 1 μg of oligomycin/mg of mitochondrial protein. Upon addition of oligomycin to the mitochondrial suspension, ATP_{in} dropped from 17 ± 3 to 1.51 ± 0.1 nmol/mg. This resulted in a dramatic fall of the translocase flux from 960 ± 55 nmol/min·mg of mitochondrial protein to levels below the detectable limit (300 nmol/min·mg) imposed by the signal-to-noise ratio for these experiments.

To determine the influence of matrix ATP levels on translocase flux, mitochondrial ATP levels were varied independently of energy state prior to flux measurements. The

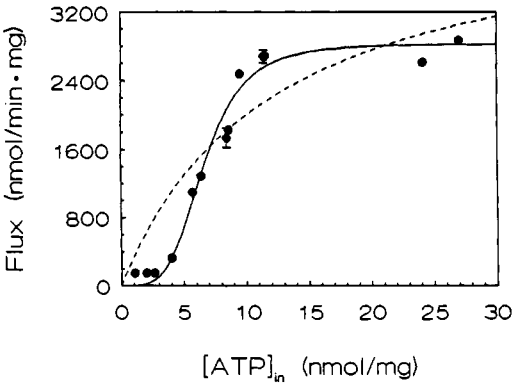

FIGURE 4: Evaluation of the effect of matrix ATP on translocase flux at 18 °C. Mitochondrial protein as well as external [ATP] was varied in order to obtain greater than 30% magnetization transfer at different levels of matrix ATP. The solid line was obtained from a nonlinear curve fit (Johnson & Frasier, 1985) using the 3-parameter Hill equation, whereas the dotted line was obtained from a 2-parameter fit using the Henri–Michaelis–Menten equation (Segel, 1976).

Mg^{2+} ATP loading method described by Aprille (1988) was used to vary intramitochondrial ATP levels over a wide range. These nucleotide levels were well maintained when extramitochondrial Ca^{2+} was removed from the resuspension buffer. Results of these experiments performed at 18 °C are shown in Figure 4. At this temperature, the plot of flux vs matrix ATP per milligram of protein was distinctly sigmoidal with a Hill coefficient of 4.07 ± 0.27. A V_{max} of 2830 ± 55 nmol/mg·min was obtained, and half-maximal flux was observed when matrix ATP levels were 6.6 ± 0.12 nmol/mg. When the matrix ATP levels were below 4 nmol/mg, it was difficult to obtain estimates. Also shown in Figure 4 is the best fit of the data obtained by assuming Michaelis–Menten single-site saturation kinetics. The K_m for this best fit line was 12.1 nmol/mg, and V_{max} was 4420 nmol/min·mg. The data do not adequately fit the curve modeling the Michaelis–Menten kinetics.

Discussion

The purpose of this study was to monitor the reactions catalyzed by the mitochondrial adenine nucleotide translocase in intact functional mitochondria. This was done to provide information regarding the kinetics of this transporter and to determine whether it plays a role in controlling the generation of ATP.

It is possible to measure the kinetics of the tranlocase by NMR saturation-transfer techniques for two reasons. First, under full relaxation, mitochondrial ATP is completely NMR-visible and the ^{31}P NMR chemical shifts of the phosphates of intramitochondrial ATP are readily resolvable from those of the extramitochondrial pools upon addition of the Mg^{2+} chelator CDTA to the mitochondrial suspension (Hutson et al., 1989). Since 95% of matrix ATP is magnesium bound (Corkey et al., 1986), removal of the divalent cation from the external environment causes an upfield chemical shift by increasing the electron shielding around the phosphorus atoms. When the intramitochondrial ATP level was increased by ATP loading (Hutson et al., 1989), the position of the intramitochondrial β-ATP spectral peak did not shift. With loading, total Mg^{2+}/ATP remained at 0.95 or higher (Mosher et al., 1991). This suggests that Mg^{2+} ATP is the substrate of the ATP/P_i "leak" transporter described by Aprille (1988). This hypothesis has been subsequently confirmed by atomic absorption measurements of intramitochondrial Mg^{2+} before and after ATP loading (Dr. Chris Doumen, unpublished observations).

A second reason for the success in making these measurements is that the rate of exchange of adenine nucleotides is faster than the spin-lattice relaxation rate for the species from which the magnetization is transferred. Therefore, rapid ATP_{out} to ATP_{in} transport is directly observable by focusing on the transfer of magnetization between the two compartments of β-ATP.

Of the classical biochemical methods for mitochondrial reaction rate determination, the inhibitor stop technique is the one most frequently used (Palmieri & Klingenberg, 1979). This approach utilizes a rapid and specific inhibitor of transport that is added at different times after the addition of an isotopically labeled substrate. The substrate, which is theoretically trapped, is then measured, and initial rates are obtained by mathematical approximation [reviewed by LaNoue and Schoolwerth (1984)]. Even if the additions are rapid and the inhibition is efficient, the best temporal resolution obtainable by this technique is in the hundreds of millisecond range for which sequential automated sampling techniques were especially developed (Pfaff & Klingenberg, 1968; Pfaff et al., 1969). Oligomycin was added to the mitochondrial suspension to inhibit the ATPase so that it would not interfere with the desired measurements by converting the isotopically labeled compound to another metabolite. The assumptions that this perturbation did not affect the measurement were probably incorrect. The reasons for this are given below.

First, results obtained from the Arrhenius plot clearly confirm the steep temperature dependence of adenine nucleotide transport (ATP–ADP) originally measured by Pfaff et al. (1969) and then again by Klingenberg et al. (1975). Unlike the absolute rates of transport, the enthalpy values are very similar to those previously reported (Klingenberg et al., 1976). Most interesting is that the data indicate that exchange of ATP_{in} for ATP_{out} is more than an order of magnitude faster than rates that have been reported previously which were obtained by using isotopic techniques in the presence of oligomycin (Klingenberg et al., 1980). The difference is ascribed partially to low levels of matrix ATP in oligomycin-inhibited mitochondria. We have demonstrated that the transporter is sensitively controlled by the availability of internal ATP. When levels of intramitochondrial ATP fall below ~8 nmol/mg of mitochondrial protein, the rate of ATP_{out} to ATP_{in} transport decreases dramatically. The sensitivity of the saturation-transfer rates to biochemically measured intramitochondrial ATP provides additional evidence that flux through the transporter is measured.

The curve relating the intramitochondrial matrix ATP level to flux is sigmoidal. The reason for the sigmoidicity is not clear, but the data are reminiscent of the results obtained by LaNoue et al. (1981). In that study the relationship between matrix ATP and flux was assessed in cardiac mitochondria by a different technique, which did not employ isotopic exchange. The undirectional electrophoretic exchange of ATP_{in} for ADP_{out} was measured by assessing net ATP synthesis as a function of matrix ADP, when external ADP was saturating.

The advantage of using ^{31}P NMR saturation-transfer techniques to study the in situ kinetics of the reactions catalyzed by the mitochondrial adenine nucleotide translocase has been demonstrated. Interesting differences in the reaction rates determined by NMR from those obtained by the classical biochemical methods have been shown. Measured rates of transport are also almost an order of magnitude faster than the ATPase (ATP/P_i) exchange rates, observed in the steady-state condition (state 4) in the absence of added ADP (LaNoue et al., 1986). Despite the high values of V_{max} ob-

served for the exchange of ATP_{in} for ATP_{out} measured in the present study, the transporter may limit net synthesis of ATP under more physiological conditions when matrix ATP is lower than in state 4 and when medium ADP is present at higher concentrations. Several recent reports (Jacobus et al., 1982; From et al., 1990) suggest that the external ratio of ATP/ADP is unlikely to regulate net ATP synthesis. Moreover, free ADP in the tissue appears to be too high to regulate synthesis via the adenine nucleotide translocase. If high ADP levels inhibit ATP entry, ADP_{in}/ADP_{out} exchange may be high and the productive exchange of external ADP for internal ATP may be low and equal to net ATP synthesis. In that case mitochondrial ATP levels would regulate ATP appearance in the cytosol with the sigmoidal kinetics observed in this study. Intramitochondrial ATP could in turn be regulated either by substrate availability as suggested by some (Katz et al., 1987) or by allosteric effects on ATP synthase as suggested by others (Moreno-Sanchez et al., 1989).

ACKNOWLEDGMENTS

We thank Richard W. Briggs and Kevin Brindle for helpful discussions and for help with the preliminary experiments. We also thank Chris Doumen for help with the final manuscript.

REFERENCES

Alger, J. R., & Shulman, R. G. (1984) *Q. Rev. Biophys. 17*, 83–124.

Aprille, J. R. (1988) *FASEB J. 2*, 2547–2556.

Austin, J. J., & Aprille, J. R. (1984) *J. Biol. Chem. 259*, 154–160.

Barbour, R. I., & Chan, S. H. P. (1981) *J. Biol. Chem. 256*, 1940–1948.

Bittl, J. A., DeLayre, J., & Ingwall, J. S. (1987) *Biochemistry 26*, 6083–6090.

Brindle, K. M., & Campbell, I. D. (1987) *Q. Rev. Biophys. 19*, 159–182.

Brindle, K. M., Blackledge, M. J., Challiss, R. A. J., & Radda, G. K. (1989) *Biochemistry 28*, 4887–4893.

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

Burat, M. K., Burat, T., Davis-van Thienen, W. I. A., & Davis, E. J. (1984) *Arch. Biochem. Biophys. 235*, 150–158.

Campbell-Burk, S. L., den Hollander, J. A., Alger, J. R., & Shulman, R. G. (1987) *Biochemistry 26*, 7493–7500.

Chance, B. C., & Williams, G. R. (1956) *Adv. Enzymol. Relat. Subj. Biochem. 17*, 65–134.

Corkey, B. E., Duszynski, J., Rich, T. L., Matschinsky, B., & Williamson, J. R. (1986) *J. Biol. Chem. 261*, 2567–2574.

Davis, E. J., & Davis-van Thienen, W. I. A. (1978) *Biochem. Biophys. Res. Commun. 83*, 1260–1266.

Degani, H., Alger, J. R., Shulman, R. G., Petroff, O. A. C., & Prichard, J. W. (1987) *Magn. Reson. Med. 5*, 1–12.

Duyckaerts, C., Sluse-Goffart, C. M., Fux, J.-P., Sluse, F. E., & Liebecq, C. (1980) *Eur. J. Biochem. 106*, 1–6.

Ericinska, M., & Wilson, D. F. (1982) *J. Membr. Biol. 70*, 1–14.

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

Freeman, R., Hill, H. D., & Kaptein, R. (1972) *J. Magn. Reson. 7*, 82–87.

From, A. H. L., Zummer, S. D., Michurski, S. P., Mohanakrishna, P., Ulstad, V. K., Thoma, W. J., & Ugurbil, K. (1990) *Biochemistry 29*, 3731–3743.

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

Heldt, H. W. (1966) in *Regulation of Metabolic Processes in Mitochondria* (Tager et al., Eds.) p 51, Elsevier, Amsterdam.

Heldt, H. W. (1967) in *Mitochondrial Structure and Compartmentation* (Quagliariello et al., Eds.) Adriatica Editrice, Bari, Italy.

Heldt, H. W., & Klingenberg, M. (1968) *Eur. J. Biochem. 4*, 1–8.

Hsieh, P. S., & Balaban, R. S. (1988) *Magn. Reson. Med. 7*, 56–64.

Hutson, S. M., Berkich, D. A., Williams, G. D., LaNoue, K. F., & Briggs, R. W. (1989) *Biochemistry 28*, 4325–4332.

Jacobus, W. E., Moreadoth, R. W., & Vandegaer, K. M. (1982) *J. Biol. Chem. 257*, 2397–2402.

Johnson, M. L., & Frasier, S. G. (1985) in *Methods in Enzymology: Nonlinear Least-Squares Analysis* (Hirs, C. H. W., & Timasheff, S. N., Eds.) Vol. 117, pp 301–342, Academic Press, London.

Katz, L. A., Koretsky, A. P., & Balaban, R. S. (1987) *FEBS Lett. 221*, 270–276.

Kemp, A., Jr., Groot, G. S. P., & Reitsma, H. J. (1969) *Biochim. Biophys. Acta 180*, 28–34.

Klingenberg, M. (1975) in *Energy Transduction Mechanism* (symposium, Stockholm, 1973) Associated Scientific Publishers, Amsterdam.

Klingenberg, M. (1976) in *The Enzymes of Biological Membranes: Membrane Transport* (Martinosi, A. N., Ed.) Vol. 3, pp 383–438, Plenum, New York.

Klingenberg, M. (1980) *J. Membr. Biol. 56*, 97–105.

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.

Kuchel, P. W. (1990) *Nucl. Magn. Reson. Biomed. 3*, 102–119.

Kuhn, W., Offermann, W., & Liebritz, D. (1986) *J. Magn. Reson. 68*, 193–197.

Kunz, W., Bohnensack, R., Bohme, G., Kuster, U., Letko, G., & Schonfeld, P. (1981) *Arch. Biochem. Biophys. 209*, 219–299.

LaNoue, K. F., & Schoolwerth, A. C. (1984) in *New Comprehensive Biochemistry, Bioenergetics* (Ernster, L., Ed.) Vol. 9, pp 221–268, Elsevier, Amsterdam.

LaNoue, K. F., Watts, J. A., & Koch, C. D. (1981) *Am. J. Physiol. 241*, H663–H671.

LaNoue, K. F., Jeffries, F. M. H., & Radda, G. K. (1986) *Biochemistry 25*, 7667–7675.

Moreno-Sanchez, R., Hague, B. A., & Hansford, R. G. (1990) *Biochem. J. 268*, 421–428.

Morris, G. A., & Freeman, R. (1978) *J. Magn. Reson. 29*, 433–462.

Mosher, T. J., Williams, G. D., Doumen, C., LaNoue, K. F., & Smith, M. B. (1991) *Magn. Reson. Med.* (in press).

Palmieri, F., & Klingenberg, M. (1979) *Methods Enzymol. 56*, 279–301.

Pfaff, E., & Klingenberg, M. (1968) *Eur. J. Biochem. 6*, 66–79.

Pfaff, E., Heldt, H. W., & Klingenberg, M. (1969) *Eur. J. Biochem. 10*, 484–493.

Rees, D., Smith, M. B., Harley, J., & Radda, G. K. (1989) *Magn. Reson. Med. 9*, 39–52.

Schneider, W. C., & Hogeboom, G. H. (1950) *J. Biol. Chem. 183*, 123–128.

Segel, I. H. (1976) in *Biochemical Calculations*, pp 309, John Wiley and Sons, New York.
Ugurbil, K. (1985) *J. Magn. Reson. 64*, 207-219.
Verhoeven, J., Kramer, P., Groen, A. K., & Tager, J. M. (1985) *Biochem. J. 226*, 183-192.
Vignais, P. V. (1976) *Biochim. Biophys. Acta 456*, 1-38.
Williamson, J. R., & Corkey, B. E. (1969) *Methods Enzymol. 13*, 434-513.
Zweier, J. L., & Jacobus, W. E. (1987) *J. Biol. Chem. 262*, 8015-8021.

Similarities in Structure between Holocytochrome b_5 and Apocytochrome b_5: NMR Studies of the Histidine Residues†

Cathy D. Moore, Ousaima N. Al-Misky, and Juliette T. J. Lecomte*
Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802
Received April 1, 1991; Revised Manuscript Received June 13, 1991

ABSTRACT: The properties of the six histidine residues of apocytochrome b_5 have been investigated by using one- and two-dimensional proton NMR spectroscopy in order to probe the structure remaining after heme removal. Spectral assignments were arrived at by analyzing proton NOE connectivities, comparing them to those observed in the holoprotein, and inspecting the X-ray structure of the latter species. Each histidine residue was studied for its pK_a value, interaction with the relaxation agent copper nitrilotriacetic acid, and reactivity toward bromoacetic acid. The four histidines which are not coordinated to the iron atom in the holoprotein (His-15, -26, -27, and -80) display in the major conformer of the apoprotein the same characteristic properties as in the holoprotein. Three of them are involved in specific interactions with the rest of the structure: His-15 and His-80 participate in hydrogen bonds, and His-27 is influenced by the nearby C-terminal segment. His-26 is the most exposed to the solvent. His-63 and His-39, which are located in the heme binding site, have distinct pK_a values; they are affected differently by the copper agent and exhibit comparable reactivity toward bromoacetic acid, albeit milder than that of His-26. The results show that the heme binding residues are clearly distinguishable by their physicochemical properties and that several elements of native holoprotein structure are in place in the apoprotein. It is proposed that the structural influence of the heme is localized and that the amino- and carboxy-terminal segments form a structural unit providing stability to the apoprotein and supporting a fluctuating, partially folded binding site.

The heme-containing, water-soluble fragment of cytochrome b_5 (cyt b_5)[1] is a small globular protein made of both α-helices and β-sheet (Mathews et al., 1971). Its heme prosthetic group is buried in the protein matrix and is known to play an essential role in the definition and stabilization of the structure of the holoprotein (Huntley & Strittmatter, 1972). In order to probe this role, we are currently characterizing physicochemical properties of the apoprotein by nuclear magnetic resonance spectroscopy. We recently reported results demonstrating the presence in the apoprotein of a stable cluster of side chains which overlaps with the second hydrophobic core of the holoprotein (Moore & Lecomte, 1990). This structured region contains the only tryptophan residue, Trp-22, and two of the protein's six histidine residues, His-15 and His-80. In the holoprotein, His-15 and His-80 are found on opposite sides of the β-sheet, form hydrogen bonds with backbone atoms, and are thought to stabilize helices I and VI, respectively (Mathews et al., 1979). The other four histidine residues are located at positions 26, 27, 39, and 63. His-26 and -27 are situated in a turn connecting strands 3 and 4; His-39 and His-63 are the two axial ligands of the iron atom and terminate two of the four helices forming the heme cavity. Thus, the six histidine residues are scattered throughout the protein and provide good markers of structural features. In particular, the two heme pocket histidine residues are expected to report on the state of the vacant binding site.

The assignment of the six histidines in rat liver apocyt b_5 is readily achieved by applying two-dimensional proton NMR techniques. To probe the environment of each residue, acid-base and paramagnetic relaxation agent titrations were performed. Histidine modification studies were also undertaken to determine relative reactivity. The results can be directly compared to those obtained in the holoprotein: the pK_a's of the histidine residues for two different isotypes of cyt b_5, beef and rabbit, are available (Altman et al., 1989) while beef liver cyt b_5 has been titrated with the relaxation agent Cu(NTA)$^-$ for the purpose of electron-transfer studies (Reid et al., 1987). Chemical modification of the histidines using DEP has also been examined (Konopka & Waskell, 1988a,b; Altman et al., 1989).

In this paper, we discuss the properties of the histidine residues of apocyt b_5 as they contribute to a detailed description of the apoprotein. The data continue to show a native holo-

† Supported in part by BRSG Grant S07 RR07082-22 awarded by the Biomedical Research Support Grant Program of the National Institutes of Health and in part by Grant DK 43101 from the National Institutes of Health.
* To whom correspondence should be addressed.

[1] Abbreviations: apocyt b_5, apo form of the water-soluble fragment of cytochrome b_5; CD, circular dichroic; DEP, diethyl pyrocarbonate; COSY, two-dimensional correlated spectroscopy; cyt b_5, water-soluble fragment of cytochrome b_5; DQF-COSY, double-quantum-filtered COSY; holocyt b_5, holo form of the water-soluble fragment of cytochrome b_5; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser spectroscopy; NTA, nitrilotriacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TOCSY, total correlation spectroscopy; 2Q, two-quantum spectroscopy.

0006-2960/91/0430-8357$02.50/0