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Saturation-transfer studies of ATP-P_i exchange in isolated perfused rat liver

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The rate of exchange between inorganic phosphate and ATP was measured in isolated perfused rat livers in the direction of ATP synthesis using ^{31}P NMR spectroscopy and the saturation-transfer technique. Measurement of ATP hydrolysis was not observable, even after treatment of rats with 100 μg T_3/day per 100 g body wt. When the perfused livers were treated with iodoacetate in order to inhibit glycolysis, NMR measurable exchange between ATP and P_i was eliminated. It is concluded that the inorganic phosphate → ATP conversion detected by saturation transfer is catalyzed by enzymes of the glycolytic pathway and that the mitochondrial ATPase rate is too slow to contribute to the observed effect.

Introduction

Saturation-transfer [1] and multiple-saturation-transfer [2,3] techniques of nuclear magnetic resonance spectroscopy (NMR) can be used to measure exchange rates between metabolic pools in the intact cells and tissues. Rates of ATP synthesis have been determined in *Escherichia coli* [4], yeast [5,6], myocardium [7,9], muscle [10] and kidney [11,12]. The rate of ATP hydrolysis has also been measured in myocardium [13]. In this study, we report the measurement of the ATP $\rightleftarrows$ P_i exchange rate in the direction of ATP synthesis in isolated perfused rat liver, obtained from normal rats, and rats treated with 3,3′,5′-triiodo-1-thyronine (thyroid hormone-T_3) in order to enhance the hydrolysis of ATP due to an increase in the activity of the cell membrane Na^+/K^+ ATPase [14]. In addition, the contribution of the glycolytic enzymes to the observed exchange was examined by use of iodoacetate, an inhibitor of glyceraldehyde-3-phosphate dehydrogenase.

Materials and Methods

Bovine serum albumin and thyroid hormone T_3 were purchased from Sigma. Reagents for the measurement of O_2 content of the perfusate were purchased from American Scientific Products. 18-h fasted rats weighing 126–150 g were anesthetized with pentobarbital. The liver was perfused in situ in the manner of Miller [15]. The inflow was through the portal vein and the outflow was through the inferior vena cava. Both the inflow and outflow vessels were cannulated, and the perfusion rate was set at 2 ml/min per g wet weight. The liver was then placed in a standard 20 mm NMR tube; 0.9% NaCl was added into the sample tube to maintain the liver within an aqueous

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environment of appropriate ionic strength and osmolarity. In this arrangement, the perfusate is only present within the vasculature of the liver; consequently, the volume occupied by the perfusate is small relative to the volume of the liver and all ^{31}P signals predominantly arise from the liver. By using a phosphorylated compound (creatine phosphate) that cannot traverse the cell membranes in liver, the perfusate volume was measured to be approx. 10% of the total volume seen by the coil.

The perfusate contained 115 mM NaCl, 5.8 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $NaSO_4$, 2.5 mM $CaCl_2$, 25 mM Na_2CO_3, 3 mM lactate, 3% dialyzed bovine serum albumin and washed rejuvenated human erythrocytes [16]; erythrocyte content was adjusted so that the final hematocrit of the perfusate was 25%. Given this hematocrit and the fact that within the detection coil, the volume occupied by the perfusate compared to the liver volume is very small in our preparation, ^{31}P signals from the erythrocytes do not contribute the ^{31}P NMR signals detected. The perfusate was oxygenated by passage through silastic tubing (0.58 mm inner diameter, 0.77 mm outer diameter, Dow Corning No. 602-235) coiled around a brass water-jacket maintained at 37° C. A gas mixture containing 95% O_2/5% CO_2 was continuously passed over the silastic tubing. The pH of the perfusate was adjusted to 7.4 after equilibration with the gas mixture. Liver was maintained at 37° C within the magnet during the NMR measurements by passing air through the NMR probe that contained the sample; the temperature of the air was regulated using the instrument's temperature regulator.

Exposure of liver to iodoacetate. Livers were exposed to iodoacetate transiently. 2 mM iodoacetate was included in the liver perfusate for approx. 15 minutes; subsequently, perfusion was switched to iodoacetate-free perfusate. Saturation-transfer measurements were conducted after the liver had stabilized from the inhibition of glycolysis, and intensities of resonances detected in the ^{31}P NMR spectra achieved a steady state.

T_3 treatment of animals. Rats were made hyperthyroid by intraperitoneal injection of 100 μg of T_3/100 g body weight per day for six consecutive days. Body weights of the rats were not increasing at the end of the injection period. The last injection was given approx. 24 h before the animal was killed.

NMR conditions and treatment of data. ^{31}P NMR spectra were obtained at 146.1 MHz using a modified Nicolet Helmholtz phosphorus probe. The broad lipid resonance was removed using a presaturating irradiation as described previously [17].

The procedure for the saturation-transfer measurements was essentially identical to that described [9], except that one low-power rf irradiation was used to suppress the broad resonance, while a second low-power rf irradiation saturated the ATPγ selectively. The progressive-saturation method was used to determine the T_1 of the P_i resonance while ATPγ magnetization was nulled [9].

A fully relaxed spectrum which consisted of 32 FIDs recorded with 90° pulses and a 20 s interpulse delay was obtained prior to each saturation-transfer measurement. The saturation-transfer data, which contained a control spectrum and a progressive-saturation measurement conducted in the presence of ATPγ saturation, were time-averaged by cycling 20-times through the entire set of repetition times, accumulating 12 scans per spectrum at each cycle. The saturation-transfer measurements were followed by the accumulation of a second set of fully relaxed spectra. The fully relaxed spectra recorded prior to and subsequent to the saturation-transfer experiments were used to calculate the concentration of free P_i and ATP in the livers; in these calculations, the resonance of 100 mM phenylphosphonate that was contained in a capillary tube was used as a reference. ADP levels were determined from fully relaxed spectra by comparing the integrated areas of the β-ATP resonance and the resonance at approx. −5 ppm which contains contributions from both ADPβ and ATPγ phosphates.

O_2 measurements. Oxygen consumption by the liver was determined by measuring the O_2 content of the perfusate before and after passage through the liver. Aliquots of the perfusate were removed from the inflow and outflow lines of the perfusion circuit and were immediately injected into a separate chamber, where they were diluted 10-fold with a hemolyzing ferricyanide solution. The O_2 was displaced from the hemoglobin by the ad-

dition of cyanide [18]. The O_2 content of the solution was subsequently measured with a Yellow Springs Instruments oxygen electrode.

The O_2 content of the erythrocyte containing perfusate was in the range of 5.6–6.3 μmol O_2/ml for the inflow and 4.9–5.7 μmol O_2/ml subsequent to passage through the liver. Specific numbers for the three groups studied are given in Table I.

Results and Discussion

The exchange observed between inorganic phosphate and the terminal phosphate of ATP by saturation transfer in the isolated perfused rat liver is demonstrated in Fig. 1. Spectrum (a) shows the phosphorus spectrum obtained when the selective irradiation was applied downfield from inorganic phosphate, so that the frequency separation from P_i was equal to the separation between inorganic phosphate and ATPγ. Spectrum (b) shows the phosphorus spectrum obtained when the ATPγ resonance was saturated. Spectrum (c) shows the difference between spectra (a) and (b). In the particular liver spectrum shown, the fractional reduction in the P_i magnetization was 12% of the intensity of the original inorganic phosphorus resonance. The P_i signal detected arises exclusively from intracellular P_i in the liver; as outlined in Materials and Methods, in our perfused liver preparation where both the inflow and outflow were cannulated, the perfusate is present only within the liver vasculature and occupies a small volume (approx. 10%) relative to the volume of the liver within the coil. Therefore, erythrocytes (contained in the perfusate) that can potentially provide a contaminating source of P_i signals did not contribute to the P_i resonance observed at the signal-to-noise ratio of our spectra.

The kinetic and metabolic data obtained from six livers are given in Table I. The average O_2 consumption rate measured for the six (normal) livers was 81 nmol/s per g dry wt. which, given the dry wt./wet wt. ratio of 4 measured for these livers, is equal to 73 μmol/h per g wet wt. the O_2 consumption rate of perfused rat livers were previously reported to be 100–150 μmol/h per g wet wt. [19]. In view of the difficulties involved in O_2 uptake measurements when erythrocytes are used

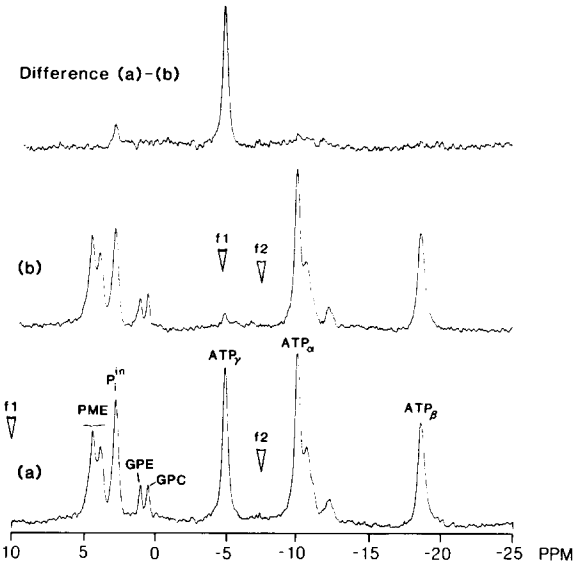

Fig. 1. ATP → P_i saturation transfer in the normal liver at 148.1 MHz. (a) Liver ^{31}P NMR spectrum with selective rf irradiation *f*1 positioned downfield from P_i, at a frequency separated from the P_i resonance by the frequency difference between P_i and ATPγ. (b) The result of saturation the ATPγ resonance. The difference spectrum (c) shows the transfer of magnetization from ATPγ to P_i. *f*1 and *f*2 designate the two separate selective irradiation frequencies used to saturate ATPγ and the broad baseline resonance. PME, phosphomonoesters; P_i^{in}, intracellular phosphate; GPC, phosphoglycerocholine; GPE, phosphoglyceroethanolamine. The resonances at approx. −5 and −10 ppm contain contributions from both ATP and ADP; since the dominant contribution is ATP, they were labeled as such.

in the perfusate, the O_2 consumption values we obtained are not very different from those reported earlier. It should also be noted that the conclusions reached in this paper are not altered even if we assume that our O_2 consumption rates were underestimated by a factor of 2; this is discussed in greater detail further on.

Recent experiments involving yeast have demonstrated a dependence on the rate of observed exchange between inorganic phosphate and ATP on the inhibition of glycolysis [6,20] at the level of glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase. Similarly, in the rat myocardium, it has recently been shown that glyceraldehyde-3-phosphate dehydrogenase/3-phosphoglycerate kinase activity contributes significantly at low workloads to the P_i → ATP rate measured by saturation transfer [21,22]. Therefore,

TABLE I

THE KINETIC AND METABOLIC DATA ON UNIDIRECTIONAL ATP SYNTHESIS RATE OBTAINED BY SATURATION TRANSFER

All values are means ± S.E. k_1 and flux were calculated for each measurement and then averaged to obtain the mean and S.E. $\Delta M/M^0$ is the fractional reduction of P_i intensity upon saturation of ATPγ. T_1* designates the T_1 of P_i measured, while ATPγ was saturated. Flux is the unidirectional rate of P_i incorporated into ATP; this is equal to k_1 [P_i]. Dry wt./wet wt. ratio was measured to be 4. For the O_2 consumption rate the content of the inflow and the effluent was 6.3 ± 0.5 and 5.7 ± 0.4 μmol O_2/ml (flow rate 14.7 ± 0.7 ml/min) for normal, 5.50 ± 0.06 and 5.15 ± 0.08 μmol O_2/ml for IA-treated (flow rate 14.4 ± 0.4 ml/min) and 5.58 ± 0.07 and 4.9 ± 0.3 μmol O_2/ml (flow rate 14.4 ± 0.4 ml/min) for the T_3-treated livers, respectively. n.d., not detectable, n.m., not measured.

Liver conditions	$\Delta M/M^0$	T_1* (s)	k_1 (s^{-1})	ATP content (μmol per g dry wt.)	P_i content (μmol per g dry wt.)	Flux (μmol/s per g dry wt.)	O_2 consumption rate (nmol O_2/s per g dry wt.)
Normal ($N = 6$)	0.175 ± 0.009	0.56 ± 0.02	0.338 ± 0.025	12.6 ± 0.6	12.6 ± 0.8	4.26 ± 0.27	81 ± 5
Iodoacetate-treated ($N = 4$)	n.d.	n.m.	n.d.	9.3 ± 0.8 [a]	19.7 ± 2.8	–	60 ± 3
T_3-treated ($N = 3$)	0.160 ± 0.016	0.43 ± 0.02	0.378 ± 0.051	12.1 ± 0.8	13.8 ± 1.2	4.57 ± 0.50	96 ± 7

[a] Calculated from the ATP and P_i content determined for normal livers and the fractional changes measured for the ATP and P_i resonance intensities in response to IA treatment. The values given correspond to ATP and P_i levels during the steady-state attained subsequent to transient IA exposure.

we examined the effect of inhibition of glyceraldehyde-3-phosphate dehydrogenase by iodoacetate on the transfer of saturation from ATPγ to P_i in the isolated perfused rat liver. Saturation-transfer experiments were repeated subsequent to exposure of the liver to iodoacetate. Addition of iodoacetate to the perfusion media induced transient increases in the sugar phosphate and inorganic phosphate levels, and caused a transient decrease in level of nucleoside di- and triphosphates in the liver. However, subsequent to exposure of IA, new steady-state levels of metabolites detected by ^{31}P NMR were established and retained throughout the following saturation-transfer measurements. For the four livers studied, the ratio of the ATP and P_i levels attained during the new steady state to the levels displayed prior to exposure to IA were 0.74 ± 0.05 (S.E.M.) and 1.56 ± 0.2 (S.E.M.), respectively. Although the total nucleotide pool decreased, the ratio of ADP/ATP remained the same. These effects are consistent with the inhibition of glyceraldehyde-3-phosphate dehydrogenase.

Saturation-transfer measurements conducted in livers exposed to iodoacetate did not show any transfer of saturation from ATPγ to the inorganic phosphate resonance (Fig. 2). At the level of our signal-to-noise ratio, IA treatment must have reduced the $P_i \rightarrow$ ATP rate approx. 3-fold or more to render the saturation transfer undetectable. A corresponding decrease of this magnitude was not noted in the O_2 consumption rate (Table I).

Livers from T_3-treated rats were examined in

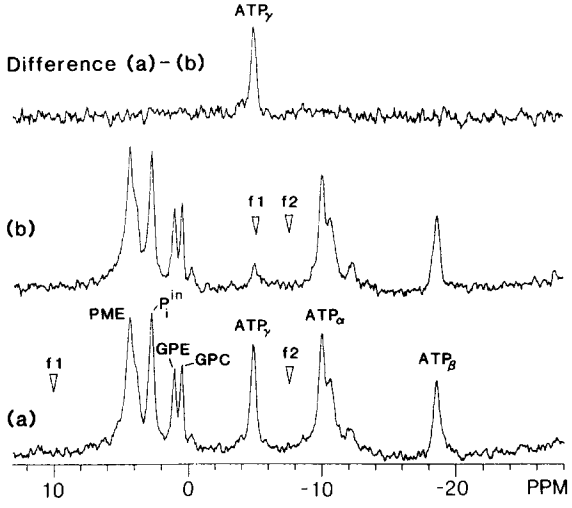

Fig. 2. Saturation transfer after iodoacetate treatment. (a) liver ^{31}P spectrum with selective r.f. irradiation downfield from P_i. (b) The result of saturation of ATPγ. The difference spectrum shows no transfer of magnetization above the signal-to-noise of the spectra.

the hope that T_3 treatment can lead to enhanced O_2 consumption rate and higher ATP synthesis rate by oxidative phosphorylation. Although the mean O_2 consumption rate was 20% higher in T_3-treated rats relative to control, the difference was not statistically significant. Clearly, the $P_i \rightarrow$ ATP rate determined by saturation transfer was not altered in T_3-treated livers. Again, iodoacetate treatment of the T_3-treated livers abolished the transfer of saturation from ATPγ to P_i without inducing a commensurate reduction in the O_2-consumption rate.

The effect of iodoacetate on the liver can be complex. However, as previously mentioned, after exposure to iodoacetate, the nucleotide pools and the O_2 consumption rate reached a new steady-state and did not change during the subsequent measurement. Therefore, it can be assumed that iodoacetate did not induce any significant uncoupling or inhibition of the oxidative-phosphorylation process. The elimination by iodoacetate of the saturation-transfer effect on P_i when ATPγ is irradiated, implies that the exchange between inorganic phosphate and ATPγ is primarily due to glyceraldehyde-3-phosphate dehydrogenase/ 3-phosphoglycerate kinase couple; the ATP $\rightleftarrows$ P_i exchange mediated by the mitochondrial H^+-ATPase is not observed within the signal-to-noise ratio of this system.

Saturation-transfer technique measures unidirectional rates of an exchange process. For the oxidation-phosphorylation process specifically, the origin of the saturation-transfer effect is complex; the rate that would be measured by saturating all ATPγ spins in the cell and monitoring cytoplasmic P_i is the unidirectional rate at which cytoplasmic P_i is converted to ATP by the activities of the mitochondrial P_i-transport and the H^+-ATPase enzymes [22]; if one or both of these reactions are operating far out of equilibrium in the well-oxygenated liver, the unidirectional rate measured is equal to the net * rate of ATP synthesis in the liver [22]. If the P_i signal that is monitored by ^{31}P NMR is of mitochondrial origin, then it is the H^+-ATPase activity that would be measured by the saturation-transfer experiments. If the P_i exchange across the mitohcondrial membrane is fast relative to the T_1 of P_i, then the activity monitored by the saturation-transfer experiments in the overall oxidative-phosphorylation process would also be due to the H^+-ATPase irrespective of whether the P_i pool detected is mitochondrial, cytoplasmic or both. If this enzyme is operating unidirectionally, the rate measured by NMR is equal to the net rate of ATP synthesis. A more detailed discussion of this general issue is presented by Uğurbil et al. [22].

In the perfused rat liver, the net rate of ATP synthesis calculated from the O_2 consumption data ** is small compared to the spin relaxation rate of P_i. The T_1 of the intracellular P_i resonance in the absence of the ATP $\rightleftarrows$ P_i exchange can be calculated from the T_1^* and k_1 values listed in Table I; it is 0.63 ± 0.05 s in the normal liver at 146 MHz. Using this spin-lattice relaxation rate, the expected fractional reduction in the P_i resonance intensity upon saturation of the ATPγ spins can be derived for any given $P_i \rightarrow$ ATP rate provided that the P_i content is known. For example, if the P : O ratio is assumed to be 3, the O_2 consumption rate given for the normal liver in Table I corresponds to a net $P_i \rightarrow$ ATP rate due to oxidative-phosphorylation of 0.49 μmol/s per g dry wt.; the pseudo-first-order rate constant, k_1, for this rate would be 0.04 s^{-1}. Consequently, the fractional reduction induced in the P_i resonance (upon saturation of the ATPγ spins) due to the net rate of ATP synthesis by oxidative phosphorylation would be only approx. 0.02. This is too small to be observed at our signal-to-noise ratio. It is possible that there exist systematic errors that have led to an underestimation of the O_2-consumption rate given in Table I; however, instead of our numbers, if we use the higher O_2 consumption rates reported earlier [19], we still obtain small fractional reductions. For an O_2-consumption rate of 150 μmol/h per g wet wt., the fractional reduction in P_i intensity due to oxidation-

* The net rate of a reaction is equal to the difference of the two unidirectional rates. In a simple two-site exchange of type A $\rightleftarrows$ B, the net rate of formation of A is equal to the unidirectional rate of formation of A from B minus the unidirectional rate at which A is reconverted to B.

** Net rate of ATP synthesis is simply equal to the product of the P : O ratio and the oxygen atom consumption rate.

phosphorylation would have been 0.048 for a P : O ratio of 3, and 0.032 for a P : O ratio of 2. These fractional reductions would have been difficult to detect at the signal-to-noise ratio of these ^{31}P NMR measurements. Therefore, if ATP synthesis by the liver mitochondria is a unidirectional process *, mitochondrial contribution to the observed $P_i \rightarrow$ ATP rate is not expected. This is consistent with the experimental observations already outlined. It would have been possible to detect a saturation-transfer effect attributable to the mitochondrial H^+-ATPase and the P_i-transport system were near equilibrium and the unidirectional rates exceeded the net oxidative ATP synthesis rate significantly.

The existence of a large contribution to saturation transfer from ATPγ to P_i by glyceraldehyde-3-phosphate dehydrogenase/ 3-phosphoglycerate kinase couple in the liver is consistent with the experimental findings that such a contribution also exists in yeast [6,20] and the rat myocardium [21,22]. In glucose perfused rat myocardium, the $P_i \rightarrow$ ATP rate determined at low workloads by NMR was found to be very high and related to the rate of oxygen atom consumption by a ratio of approx. 7 [21–23]. This observation can be explained by postulating either that the mitochondrial H^+-ATPase operates rapidly in both directions and is near equilibrium or that there exists non-oxidative contributions to the NMR measurable $P_i \rightarrow$ ATP rate. In our preliminary report on the myocardial $P_i \rightleftarrows$ ATP exchange kinetics, we favored the former explanation [23]; at the time, there did not exist any experimental evidence for or against this explanation. However, our subsequent studies [21,22] have definitively shown this explanation to be incorrect; instead, it was experimentally demonstrated that the myocardial H^+-ATPase operates unidirectionally and there exists a large and workload dependent glyceraldehyde-3-phosphate dehydrogenase/ 3-phosphoglycerate kinase contribution to the $P_i \rightarrow$ ATP rate measured by NMR in glucose-perfused hearts. As in the myocardium, liver and yeast cells, glyceraldehyde-3-phosphate dehydrogenase/ 3-phosphoglycerate kinase catalyzed $P_i \rightleftarrows$ ATP exchange may also contribute significantly to saturation-transfer measurements in other cells. Therefore, unless this activity is eliminated specifically or is shown not to exist in the particular cell type under study, the saturation-transfer measurements of the $P_i \rightarrow$ ATP rate cannot be ascribed to oxidative-phosphorylation with certainty.

Both in the yeast and in the myocardium, after specific elimination of the glyceraldehyde-3-phosphate dehydrogenase/3-phosphoglycerate kinase activity, a saturation-transfer effect attributable to oxidative phosphorylation was detectable. This clearly is not the case in the liver. Relative to the myocardium, the liver O_2 consumption rate is low and the T_1 of the P_i resonance is short; these differences can account for the lack of a measurable effect due to the oxidative phosphorylation in the liver. In the mycocardium, it was specifically shown that ATP $\rightleftarrows$ P_i reaction mediated by the mitochondrial H^+-ATPase is occurring unidirectionally in the direction of ATP synthesis [21,22]. As discussed above, the data presented in this paper also suggests that ATP synthesis by the liver mitochondria is occurring predominantly unidirectionally and that the H^+-ATPase is far out of equilibrium.

Previously, an attempt to measure the liver $P_i \rightarrow$ ATP exchange rate was made in whole rats [23]. However, a saturation transfer from ATPγ to P_i was not detected. This is probably due to the lower signal-to-noise ratio of the in vivo measurements. An additional complication encountered in vivo is the carbon-substrate condition and the metabolic state of the liver. Since the source of the saturation-transfer effect is glycolytic, this effect will depend on the activity of the glyceraldehyde-3-phosphate dehydrogenase/ 3-phosphoglycerate kinase couple. This in turn will be influenced by the carbon substrate condition, which in vivo is not well defined.

In summary, it is shown that the chemical exchange between inorganic phosphate and the gamma phosphate of ATP can be observed in the isolate perfused rat liver using saturation transfer and that this exchange is primarily conducted by the glyceraldehyde-3-phosphate dehydrogenase/ 3-phosphoglycerate kinase enzymes. Mitochondrial

* I.e., the rate of ATP $\rightarrow$ P_i conversion by the mitochondrial H^+-ATPase and/or the P_i transport system is negligible compared to the $P_i \rightarrow$ ATP rate.

synthesis or hydrolysis of ATP could be examined by saturation transfer suggesting that ATP synthesis by the mitochondrial H^+-ATPase occurs virtually unidirectionally.

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