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Occurrence of an Uncoupler-Resistant Intermediate Type of Phosphate–Water Oxygen Exchange Reaction Catalyzed by Heart Submitochondrial Particles†

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ABSTRACT: The hydrolysis of ATP catalyzed by phosphorylating vesicles prepared from bovine heart mitochondria by ultrasonic disruption was studied in $H_2{}^{18}O$. Provided that an ATP-regenerating system was included to prevent accumulation of ADP due to hydrolysis, the addition of 20 mM arsenate or 0.5 mM 2,4-dinitrophenol to the incubation mixture either singly or together, had little or no effect on the number of oxygen atoms from H_2O incorporated (on the average) into each molecule of P_i formed by hydrolysis (the O:P ratio). As the ATP concentration was reduced from 2.0 to 0.05 mM, the O:P ratio increased from about 1.4 to over 2.0 and, although dinitrophenol significantly increased the ATPase activity, it did not significantly alter the O:P ratio for a given ATP level. This implies that the uncoupler does not act directly on the terminal transphosphorylation step. Companion experiments were performed in which ^{18}O label was placed either initially in H_2O or P_i. Under conditions where extensive exchange from $H_2{}^{18}O$ into P_i occurred, no ^{18}O was lost from medium P_i under identical circumstances, thus showing that the exchange was intermediate and did not involve medium P_i. Kinetic plots of v vs. v/S were nonlinear with respect to ATPase activity. The kinetic data, as well as the $P_i = H_2{}^{18}O$ exchange data, are consistent with enzyme models having multiple forms of catalytic sites. Several models are evaluated and attempts are made to distinguish between some of the simpler cases of these models.

Cohn (1953) first reported evidence for a mitochondrial catalyzed pathway whereby oxygen from inorganic phosphate (P_i)[1] equilibrated with water oxygen. Subsequent studies by other workers showed that mitochondria possessed the capacity to exchange ^{18}O from P_i into H_2O ($[^{18}O]P_i = H_2O$), from $[^{18}O]H_2O$ into P_i ($P_i = [^{18}O]H_2O$), and from $[^{18}O]H_2O$ into ATP ($ATP = [^{18}O]H_2O$). These reactions, like the $^{32}P_i$ = ATP exchange, occurred in the absence of net ATP synthesis by oxidative phosphorylation and appeared to be closely associated with a partial reaction catalyzed by the terminal phosphoryl transferase enzyme of oxidative phosphorylation, namely, the reversible formation of ATP by the reaction ADP + P_i = ATP + H_2O (for a comprehensive review of earlier work in this area, see Boyer, 1967).

As discussed in some detail (Boyer, 1967), a rapid interconversion of ADP, P_i, and ATP on the enzyme surface could produce high rates of either $P_i = H_2{}^{18}O$ or $ATP = H_2{}^{18}O$ exchange, when measured with respect to the rate of the $^{32}P_i$ = ATP exchange, but not both unless there is some additional means for incorporating ^{18}O from H_2O into ATP or P_i by a process not associated with the $^{32}P_i$ = ATP exchange. It was subsequently concluded that such a component might indeed be associated with the $P_i = H_2{}^{18}O$ exchange, particularly in view of the ability of an ATP regenerating system to suppress the $ATP = H_2{}^{18}O$ and $^{32}P_i$ = ATP exchange but still to permit $P_i = H_2{}^{18}O$ exchange to occur as ATP hydrolysis took place (Mitchell et al., 1967).

The residual $P_i = H_2{}^{18}O$ exchange activity catalyzed by submitochondrial heart vesicles, in the presence of an ATP-regenerating system, was found to be resistant to high concentrations of As_i by De Master and Mitchell (1973), despite the well-known ability of As_i to compete with P_i in P_i-requiring reactions. This observation suggested that medium P_i was not involved in the exchange, and that the incorporation of ^{18}O from H_2O had occurred with some enzyme-bound intermediate involved in ATP hydrolysis (i.e., an "intermediate" exchange). This type of intermediate has been observed with myosin (Levy and Koshland, 1959) and is distinct from the type involving medium P_i as a reactant (i.e., "medium" exchange). The data reported in the present paper confirm the existence of a $P_i = H_2{}^{18}O$ reaction of the intermediate type. This reaction is closely associated with ATP hydrolysis catalyzed by an oligomycin sensitive ATPase, but, unlike other ATP supported reactions of mitochondria, it is not inhibited by dinitrophenol. Moreover, as the concentration of ATP is decreased, the incorporation of ^{18}O into P_i due to the exchange increases from 40% to 200% (over that expected for simple hydrolysis). The implications of these findings in terms of heterogeneity of ATP enzyme complexes involved in ATP hydrolysis by submitochondrial particles are discussed.

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[1] Abbreviations used are: P_i, inorganic phosphate; As_i, inorganic arsenate; dinitrophenol, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

0006-2960/78/0417-0473$01.00/0

Independent studies by Boyer's group have also provided evidence for a mitochondrial-catalyzed phosphate-water oxygen exchange characterized by resistance to uncouplers (Rosing et al., 1977).

Experimental Procedures and Analysis of Data

Submitochondrial particles from bovine hearts were prepared essentially by the method of Hansen and Smith (1964) except that they were obtained prior to use from mitochondria stored at −15 °C using a Bronson sonifier with a special microtip attachment. ATPase activity, with or without As_i present, was measured using a 2-butanol/benzene extraction procedure and reading the absorbance of the phosphomolybdate complex at 310 nm (Huang and Mitchell, 1972). Submitochondrial vesicles were incubated in the reaction mixture at 25 °C for 4 to 5 min before the addition of ATP to start the reaction. Separate time course experiments were run to establish the linearity of the reaction in the presence of an ATP-regenerating system, and in addition the ATPase study was repeated using a continuous coupled spectrophotometric assay (using lactate dehydrogenase) with and without FCCP as uncoupler to yield results very similar to the single-point P_i assay data. Incubation times and volumes were arranged so that roughly equal amounts of P_i were obtained for ^{18}O analysis regardless of ATP concentration. The amount of NADH formed by reverse electron flow (Table I) was estimated on an aliquot of the total reaction mixture by measuring the increase in 340-nm absorbance using a recording spectrophotometer.

The medium atom % of $H_2{}^{18}O$ was measured on aliquots of reaction mixture obtained by distillation at reduced pressure. In some cases, it was calculated from the atom % measured for dilutions of bottle $H_2{}^{18}O$ with nonenriched H_2O, after making allowances for the sucrose concentration of the medium.

^{18}O analysis of P_i was performed on P_i isolated from reaction mixtures following termination by perchloric acid addition, removal of protein by centrifugation, and removal of samples for estimation of P_i concentration. The ^{18}O content of P_i or H_2O was determined by the guanidine hydrochloride method as described by Boyer and Bryan (1967). This procedure involves heating the sample in a sealed tube to produce CO_2, the enrichment of which was measured directly using a CEC 21-401 mass spectrometer in isotope ratio mode.

$H_2{}^{18}O$ was purchased from Miles Laboratories, and P_i of approximately 20 atom % enrichment was prepared from enriched water by heating in a sealed tube, as described by Boyer and Bryan (1967). Standard curves were obtained from five freshly prepared CO_2 samples using H_2O of 0.204 to about 1.6 atom % ^{18}O, by means of a linear regression method. The significance of the regression was <0.01 as determined by the F statistic. The standard error in calculating unknown atom % values from the regression line was about 1%.

The O:P ratio[2] was calculated as follows. A comparison of the atom % excess of ^{18}O in the P_i recovered at the end of the experiment with the atom % excess of the water of the incubation medium gives the fraction of oxygen in P_i which was replaced with oxygen from water during the incubation. If no P_i was added to the incubation mixture, i.e., if all the P_i analyzed was formed by ATP hydrolysis, then this value represents the O:P ratio. The extra oxygen (i.e., that oxygen incorporated by exchange) in the P_i pool from water is obtained by multiplying the O:P ratio by the amount of P_i formed by hydrolysis (to give total oxygen from water), and then subtracting one oxygen for every P_i formed by hydrolysis. In the case where unlabeled P_i was initially present, the total amount of oxygen from water in the P_i pool was calculated from the fractional replacement and the total P_i present at the end of the experiment. Dividing this number by the amount of P_i formed by hydrolysis gave the O:P ratio. For example, if the medium H_2O is 0.944 atom %, the isolated P_i is 0.503 atom % and the background is 0.213 atom % (see Table V), then the fractional replacement of oxygen in P_i is

$$(0.503 - 0.213)/(0.944 - 0.213) = 0.397$$

The total number of gram atoms of oxygen is therefore

$$0.397 \times 4 \times 2.2 = 3.494$$

The factor of 4 represents the number of oxygen atoms in P_i. The O:P ratio is

$$3.494/1.1 = 3.17$$

The total extra oxygen is then

$$(3.17 - 1) \times 1.1 = 2.39$$

The kinetic data were analyzed by a computer program (KICTESS) which uses methods described by Spears et al. (1971) and Osmundsen (1975). The procedure involved segregating the kinetic data into a high and a low substrate concentration block. Estimates for K_m and V_m were then made by using a nonlinear, iterative technique (Wilkinson, 1961; and Cleland, 1967). Initially, the high substrate block was used to provide estimates of kinetic constants for the high K_m enzyme, and these values were used to provide values for the contribution of this enzyme to the low substrate block velocities. This enabled the velocities and hence the kinetic constants attributed to the low K_m enzyme to be estimated, and permitted further refinement of the kinetic constants for the high K_m enzyme using corrected velocities obtained by subtraction of the low K_m enzyme contribution. The procedure was continued until the values for the kinetic constants converged to within 0.005 %[3] or until 40 iteration cycles had been achieved. All possible high and low substrate concentration blocks were tested (using at least three substrate concentrations per block) for a total of at least ten different ATP concentrations over a 300-fold concentration range. The lowest ATP concentration was selected so as to yield a significant amount of ATP hydrolysis over control incubations, i.e., incubations with the pyruvate kinase system but with no added ATP. Kinetic constants were chosen when the substrate concentration between the blocks appeared to fall in the vicinity of the inflection point as shown on an Eadie-Hofstee plot of the data and when the procedure yielded reasonable values for the kinetic constants (greater than 0.0). The final values for the constants were chosen from these sets based on the degree of convergence and on the ability of the method to minimize the residuals for any given dividing point. An alternative procedure which proved

[2] The O:P ratio is defined as the number of oxygen atoms from H_2O incorporated into P_i during the experiment, divided by the number of molecules of P_i formed by hydrolysis. For an intermediate exchange, i.e., one that occurs between enzyme-bound intermediates during the course of hydrolysis, the maximum possible ratio would be 4 since all oxygens of P_i would be replaced by oxygen from H_2O. For simple hydrolysis without exchange the value would be 1. If P_i of the medium also participates in the exchange (medium exchange), O:P ratios greater than 4 are possible. As discussed here, mitochondria are capable of catalyzing both the long-recognized medium P_i exchange and an intermediate type. If both occur simultaneously, the O:P ratio measurement does not have a precise mechanistic significance. However, if conditions are established that selectively suppress the medium exchange, as has been done for most of the experiments described here, then the O:P ratio provides information about the ATP hydrolytic step.

[3] The tolerance used in obtaining these fits is lower than the value (0.1%) used by Atkins (1971).

TABLE I: Failure of 0.5 mM 2,4-Dinitrophenol to Markedly Inhibit P_i-$H_2{}^{18}O$ Exchange Accompanying ATP Hydrolysis in the Presence of an ATP-Regenerating System.[a]

Additions or deletions	Total P_i released (μmol)	Total NADH formed (μmol)	O:P ratio	Total extra oxygen in P_i (atoms)
None	30.8	1.6	1.65	20.0
+dinitrophenol	42.2		1.61	25.7
−KCN, −succinate	24.3		1.53	12.9
−KCN, −succinate, +dinitrophenol	39.8		1.54	21.5

[a] The complete mixture contained in a final volume of 10 mL, pH 7.5, 0.25 M sucrose, 50 mM Tris-sulfate, 10 mM K_2SO_4, 10 mM $MgSO_4$, 10 mM potassium succinate, 4 mM PEP, 2 mM ATP, 1 mM KCN, and 1 mM NAD^+. Additions and deletions were as shown. Incubations were carried out for 5 min 15 s at 25 °C, in the presence of 0.16 mg mL^{-1} mitochondrial protein and 0.04 mg mL^{-1} of pyruvate kinase, in H_2O containing 0.696 atom % excess ^{18}O.

TABLE II: Effect of 0.5 mM 2,4-Dinitrophenol on $P_i = H_2{}^{18}O$ Exchange Accompanying ATP Hydrolysis in the Presence and Absence of ADP.[a]

Dinitrophenol	ADP	Total P_i released (μmol)	O:P ratio	Total extra oxygen in P_i (atoms)
−	−	9.3	1.61	5.7
−	−	8.9	1.61	5.4
+	−	11.9	1.63	7.5
+	−	12.0	1.63	7.6
−	+	5.4	2.50	8.1
−	+	5.3	2.48	7.8
+	+	12.6	1.96	12.1
+	+	13.0	1.91	11.8

[a] Incubation conditions were similar to those described for the complete system described in Table I except that 2.5 mM ATP and 0.09 mg mL^{-1} of protein were used. An ATP-regenerating system was included in the incubation mixtures when no ADP was added and omitted from incubations containing 0.5 mM ADP added initially. Samples with ADP were incubated for 10 min, and those without for 5 min.

useful when curvature of the Eadie-Hofstee plots was slight was to initiate the calculation using the low substrate velocity block. This procedure minimized the occurrence of negative partial velocities in the calculation. The rate equation for the two Michaelis-Menten type enzymes operating independently (i.e., noninteracting sites) on a common substrate (eq 1) is of the same form as that for two catalytic sites operating in an anticooperative fashion (eq 2, see Figure 1) (Segel, 1975). It is thus possible to define values for K_s, V_m, α, and β, in terms of $K_{m(l)}$, $V_{m(l)}$, $K_{m(h)}$, and $V_{m(h)}$ as shown (eq 3-6).

$$v = \frac{V_{m(l)}S}{S + K_{m(l)}} + \frac{V_{m(h)}S}{S + K_{m(h)}} \quad (1)$$

$$v = \frac{\frac{2V_m[S]}{K_s} + \frac{2\beta V_m[S]^2}{\alpha K_s{}^2}}{1 + \frac{2[S]}{K_s} + \frac{[S]^2}{\alpha K_s{}^2}} \quad (2)$$

$$K_s = \frac{2K_{m(l)}K_{m(h)}}{K_{m(l)} + K_{m(h)}} \quad (3)$$

$$V_m = \frac{V_{m(l)}K_{m(h)} + V_{m(h)}K_{m(l)}}{K_{m(l)} + K_{m(h)}} \quad (4)$$

$$\alpha = \frac{(K_{m(l)} + K_{m(h)})^2}{4K_{m(l)}K_{m(h)}} \quad (5)$$

$$\beta = \frac{(K_{m(l)} + K_{m(h)})(V_{m(l)} + V_{m(h)})}{2(V_{m(l)}K_{m(h)} + V_{m(h)}K_{m(l)})} \quad (6)$$

Consequently, kinetic constants obtained from fitting data to the two enzyme model using KICTESS can be used to calculate the kinetic constants and interaction coefficients α and β for the anticooperative model. Thus for either model it is possible to calculate for any ATP concentration the partial site velocity, i.e., the contribution that each site makes to the total velocity. The assumption that each site is characterized by an intrinsic O:P ratio (n_1 and n_2) yields a set of normal equations which can be derived from eq 7.

$$v(\text{total}) \times \text{O:P(obsd)} = n_1 p + n_2 q + \text{constant} \quad (7)$$

The values, v (total) and O:P (observed), are the experimentally determined ATPase velocity and O:P ratio for a given concentration of S (ATP). The quantities p and q are the partial velocities for the singly and doubly occupied enzyme, respectively. Statistical tests on ^{18}O data (including paired sample Student's t test on samples with and without uncoupler) were made using the statistical analysis package (SPSS release 6.02) as documented by Nie et al., (1975). Reported P values are given for the two tailed test of significance.

Results

Table I shows the occurrence of $P_i = H_2{}^{18}O$ exchange accompanying ATP hydrolysis catalyzed by heart submitochondrial vesicles in the presence of an ATP-regenerating system. The occurrence of such an exchange in the presence of an ATP-regenerating system is in accord with previous reports (Hinkle et al., 1967; Mitchell et al., 1967). The new finding shown in Table I is that the O:P ratio, which is a measure of the rate of exchange relative to the rate of hydrolysis, was not markedly decreased by the addition of dinitrophenol. Regardless of the presence or absence of uncoupler, about 60 to 65% more oxygen from water was incorporated into each P_i released by hydrolysis than would be required for simple hydrolysis. Moreover, when the stimulation of ATPase activity by uncoupler is taken into account, it can be seen that uncoupler actually stimulated the exchange since there was a larger amount of P_i formed and hence more extra oxygen in the P_i pool when uncoupler was present. The omission of KCN and succinate, which are necessary for reverse electron flow (Table I), did not markedly alter the O:P ratio.

Mitchell et al. (1975b) reported that the addition of ADP to submitochondrial vesicles actively catalyzing ATP hydrolysis resulted in an elevation of exchange relative to hydrolysis and, at the same time, rendered the phosphate-water oxygen exchange more sensitive to inhibition by As_i. They attributed these effects to the onset of an oxygen exchange reaction involving medium P_i, due to the addition of ADP. The effect of ADP on the exchange, and on the response of the exchange to dinitrophenol, was therefore studied. In these experiments the complete system for reverse electron flow was also included. As discussed later, these additional components are not obligatory for the demonstration of the effects of uncoupler on the exchange that were found. Table II confirms the observation that the O:P ratio obtained in the presence of an ATP-regenerating system was not markedly altered upon addition of uncoupler. However, when ADP and ATP were added together (and an ATP-regenerating system omitted) the O:P ratios were elevated and the ratios were now decreased upon addition of dinitrophenol.

TABLE III: Enhancement of P_i–$H_2{}^{18}O$ Exchange Relative to Rate of ATP Hydrolysis by Lowering the Concentration of ATP.[a]

ATP (mM)	ΔP_i released (mM)	O:P ratio	Total extra oxygen in P_i (mM H_2O oxygen exchanged into P_i)
2.00	5.2	1.44	2.3
2.00	5.6	1.44	2.5
0.20	2.2	1.71	1.6
0.20	2.2	1.70	1.5
0.10	1.6	1.83	1.3
0.10	1.6	1.80	1.3
0.05	1.0	2.01	1.0
0.05	1.1	2.02	1.1

[a] Incubations were carried out in medium containing 0.25 M sucrose, 50 mM Tris sulfate, 10 mM $MgSO_4$, 10 mM K_2SO_4, and 0.38 mg mL^{-1} of mitochondrial protein, at pH 7.5, for 15 min at 25 °C, in H_2O containing 0.759 atom % excess ^{18}O. A regenerating system was used to maintain ATP. Incubation volumes were 2.5, 5.0, 10.0, and 20.0 mL for mixtures containing 2.0, 0.2, 0.1, and 0.05 mM ATP respectively. The PEP concentrations ranged from 2.0 mM to 10.0 mM.

TABLE IV: Inability of As_i or Dinitrophenol (Singly or Together) to Inhibit P_i–$H_2{}^{18}O$ Exchange Accompanying ATP Hydrolysis (0.1 mM ATP) in the Presence of an ATP-Regenerating System.[a]

Inhibitor	ΔP_i released (mM)	O:P ratio	Total extra oxygen exchanged into P_i (mM H_2O oxygen exchanged into P_i)
None	1.5	1.97	1.4
None	1.4	1.92	1.3
Dinitrophenol	2.3	2.01	2.3
Dinitrophenol	2.3	1.99	2.3
As_i	1.7	1.71	1.2
As_i + dinitrophenol	2.4	1.87	2.1

[a] The complete mixture contained in a final volume of 10 mL, pH 7.5, 0.25 M sucrose, 50 mM Tris–sulfate, 20 mM $MgSO_4$, 20 mM K_2SO_4, 4 mM PEP, 0.04 mg mL^{-1} of pyruvate kinase, and 0.39 mg mL^{-1} of mitochondrial protein. Reaction was started by ATP addition and the samples were incubated for 15 min at 25 °C in H_2O containing 0.723 atom % excess ^{18}O. Concentrations of dinitrophenol and As_i were 0.5 and 20 mM, respectively.

Thus it appeared that, in the presence of ATP, the phosphate–water oxygen exchange capacity, as judged by the O:P ratio, was decreased upon addition of As_i or dinitrophenol, whereas, in the absence of ADP, the O:P ratio was not markedly decreased by As_i or uncoupler. These observations suggested that in addition to the long-established capacity of mitochondria to sustain an exchange reaction between P_i and water oxygens, using the $[^{18}O]P_i$ of the medium as a reactant (Cohn, 1953), there also exists a second type of exchange reaction which is not inhibited by As_i or dinitrophenol, and which is responsible for the prominent exchange observed in these systems when an ATP-regenerating system is used to sustain ATP hydrolysis. The properties of this new type of exchange were thus further studied, using an ATP-regenerating system to suppress the other component of exchange attributed to the participation of P_i of the medium.

Table III shows the effect of lowering the ATP concentration on the exchange reaction. In this and in subsequent experiments, the components necessary for reverse electron flow (succinate, NAD^+, and KCN) were omitted, since their presence was not necessary for appearance of uncoupler-resistant exchange. The purpose of this experiment was to see if the O:P ratio was altered when the ATP concentration was decreased below the levels required to saturate the ATPase activity. As shown, there was a marked and quite reproducible tendency for the O:P ratio to increase as the ATP concentration was lowered. It may be questioned if this is a genuine increase in an intermediate exchange or if it represents an artifact, e.g., onset of a medium exchange due to some idiosyncrasy of the regenerating system (e.g., due to an alteration of the steady-state ATP:ADP levels).

TABLE V: Demonstration of an Intermediate P_i–H_2O Oxygen Exchange Accompanying ATP Hydrolysis. Label Initially in H_2O.[a]

ATP added (mM)	P_i–$H_2{}^{18}O$ exchange: ΔP_i released (mM)	O:P ratio	Atom % ^{18}O in P_i
0.01	1.1	3.17	0.503
0.01	1.1	2.82	0.471
0.0	0.03		0.220
0.0	0.06		0.219

[a] Initial P_i concentration was 1.1 mM. ATP was hydrolyzed in the presence of 0.5 mM dinitrophenol to give approximately 2 mM final concentration. In the companion experiment the conditions were identical except ^{18}O was in the starting P_i. Incubation conditions were otherwise similar to those given in Table IV. The atom % values given for P_i from incubations without added ATP are not significantly different from the predicted background (0.213 atom %) derived from the standard curve, at the 95% confidence level. Medium water was 0.944 atom %.

Table IV shows that the exchange at low ATP concentrations retained its insensitivity to dinitrophenol or to As_i, these reagents being added either singly or together. Consequently the exchange at low ATP levels could not be attributed to medium exchange but was an enhanced form of the exchange at high ATP levels.

In order to test for the presence of a measurable medium exchange in the submitochondrial preparations under the experimental conditions used, an experiment was performed in which the source of the label was varied. Placement of the label in P_i ($[^{18}O]P_i = H_2O$ exchange) enables the medium P_i exchange contributions to be estimated. Placement of the label in H_2O enables the total exchange (medium plus intermediate) to be estimated.

Table V confirms the capacity of a submitochondrial preparation to sustain a very prominent $P_i = H_2{}^{18}O$ exchange at low ATP levels, in the presence of dinitrophenol. Part B of the table shows the results from a companion experiment performed under identical conditions, except that the initial placement of the label was in P_i. In both cases, ATP was hydrolyzed for a sufficient length of time to increase the P_i pool from 1 to about 2 mM. In Table VI, the P_i recovered at the end of the experiment was split into two parts, and each sample was separately diluted with nonenriched P_i to bring its enrichment into the range of those P_i samples measured in Table V.

The values thus obtained, when adjusted for the dilution of label due to ATP hydrolysis and nonenriched P_i addition, provide estimates of the initial enrichment of the medium P_i prior to the start of hydrolysis. Duplicate zero-time values of 20.1 and 21.0 atom % excess were obtained. Provided that no medium P_i underwent exchange, with loss of oxygen to water, the adjusted values for the incubated samples should yield

TABLE VI: Demonstration of an Intermediate P_i-H_2O Exchange Accompanying ATP Hydrolysis. Label Initially in P_i.

ATP added (mM)	ΔP_i (mM)	$[^{18}O]P_i = H_2O$ exchange: ^{18}O recovered in P_i at end of experiment (atom % excess) Duplicates	Average
0.01	1.0	20.3 21.2	20.8
0.01	1.1	19.6 20.5	20.0
0.0	0.10	22.8	22.8
0.0	0.06	20.6 20.8	20.7
Zero time		19.5 20.6	20.0
Zero time		20.6 21.5	21.0

TABLE VII: Analysis of Kinetic and Exchange Data in Terms of Two-Catalytic Site Models (Interacting or Noninteracting Sites).[a]

	No uncoupler	Plus uncoupler
	Noninteracting Sites	
K_m (high)	1.147 (0.0202)	0.515 (0.0087)
V_m (high)	0.945 (0.0053)	1.343 (0.0084)
K_m (low)	0.017 (0.0087)	0.06421 (0.000047)
V_m (low)	0.032 (0.0039)	0.10187 (0.000049)
n_1	4.6 (0.36)	5.4 (0.48)
n_2	1.094 (0.0293)	0.98 (0.051)
	Interacting Sites	
K_s	0.033	0.11
V_m	0.045	0.21
α	17.41	2.54
β	10.75	3.023
n_1	3.56 (0.247)	2.66 (0.152)
n_2	1.210 (0.0210)	1.291 (0.0202)

[a] K_m and K_s values are expressed as mM, and V_m values are given as mM min^{-1}. Standard errors are in parentheses. Constants were obtained from the analysis of data shown in Figure 2 for the hydrolysis of ATP in $H_2{}^{18}O$. Calculations were carried out using double precision arithmetic and the values are reported as recommended by Sokal and Rohlf (1969). Standard errors of the kinetic constants for the interacting site model are not included since this information cannot assist in distinguishing between the two models under consideration.

similar values. The control incubations performed in the absence of ATP were done to ensure that no exchange between medium P_i and water oxygens occurred as a result of some extraneous phosphatase activity. As can be seen in Table VI, the estimated values for the ^{18}O recovered in P_i (following incubation of labeled P_i with submitochondrial vesicles) were not markedly different from the zero-time values.

In contrast, when the label was placed initially in water, each P_i formed by hydrolysis apparently had about three oxygens from water, of which one was due to hydrolysis (as required by the stoichiometry) and two were due to exchange. Since the P_i pool increased from about 1 to 2 mM, due to ATP hydrolysis, approximately two out of every eight oxygens (i.e., about $1/4$) of the P_i pool were derived from water by exchange. Consequently, if a loss of similar magnitude were to occur from medium P_i to water, approximately 25% of the oxygen in P_i should exchange with water.

If no correction for dilution of label due to hydrolysis of ATP is applied, then about 25% of the oxygen in the medium P_i should be unaccounted for at the end of the experiment. This would correspond to the amount of ^{18}O transferred from medium P_i to H_2O. However, the initially labeled P_i was diluted with P_i due to hydrolysis, so that the *average* enrichment during the incubation was only about three-fourths of that of the starting P_i, since at the end of the incubation, the atom % excess of the P_i was decreased to one-half of its initial value by dilution with nonenriched P_i formed by hydrolysis. Consequently, the loss of ^{18}O which would be expected to occur from medium P_i, were a medium exchange to occur, would be about three-fourths of 25%, i.e., about 19%. Under the conditions employed (dinitrophenol plus an ATP-regenerating system present), no loss of this magnitude was found.

The variation of O:P with ATP concentration raised the question as to whether the exchange was catalyzed by a relatively minor ATPase activity with a low K_m for ATP, or whether the change reflects an unsuspected aspect of a single ATPase displaying negative cooperativity. Since submitochondrial particles were used in the previous experiments and in view of the contention of Astle and Cooper (1974) that these particles contain mixtures of vesicles of different sidedness, it seemed desirable to check the ATP concentration effect on a nonsonicated preparation, i.e., on washed freeze-thawed mitochondria. This study showed the O:P ratio obtained using 4 mM ATP was low (O:P = 1.3 and 1.4 in the absence and presence of uncoupler, respectively) and that the effect of reducing the ATP concentration was to increase the O:P ratio as before. With 0.02 mM ATP, the O:P ratios were 2.4 and 2.3 in the absence and presence of uncoupler, respectively. Consequently the ATP effect is not an artifact of sonication. To test for a minor ATPase activity of nonmitochondrial origin, the inhibitory effect of oubain and ruthenium red was examined at low and high ATP concentrations. No differential inhibitory effect was noted. Titration of ATPase with oligomycin resulted in progressive inhibition of ATP hydrolysis but with no marked change in the O:P ratio. All these inhibition experiments were carried out using an ATP-regenerating system to suppress the medium exchange.

To make assessments about the relationships between ATPase activity and O:P ratios in the absence and presence of uncoupler, it was necessary to carry out a series of kinetic experiments over a wide range of ATP concentrations, both in the presence and absence of uncoupler, with a view to establishing conditions for making a simultaneous determination of O:P ratio and ATPase velocity measurements. Since the equations for the two cases are of similar form, it was only necessary to analyze kinetic data using KICTESS as described in Experimental Procedures and Analysis of Data, and then to express the results either in terms of two independent ATPases, or in terms of a single negatively cooperative ATPase, with appropriate values assigned as interaction coefficients. This procedure produces the same regression line but yields different values of the constants for the two models. Eadie-Hofstee plots of such an experiment are shown in Figure 2. The kinetic constants used to obtain this fit are given in Table VII, for the two-enzyme and negative cooperativity case, respectively. An important feature of dinitrophenol reported in Tables I and II was its apparent ability to elevate ATPase without exerting pronounced effect on the O:P ratio. This feature was examined in more detail for the ^{18}O data obtained concomitantly with the ATPase data shown in Figure 2. A paired sample Student's t test was performed for pairs of data obtained in the absence or presence of dinitrophenol, using nine ATP concentrations ranging from 0.02 to 4 mM. Two re-

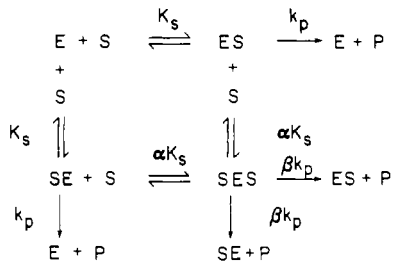

FIGURE 1: General model for two identical catalytic sites operating in an anti-cooperative fashion.

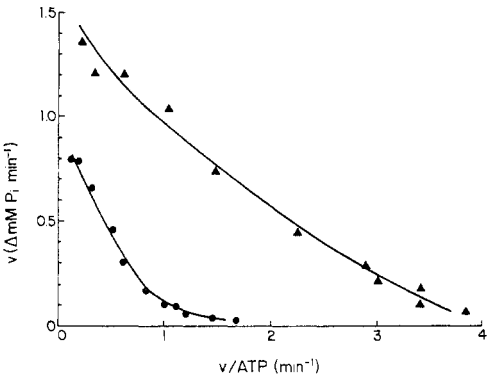

FIGURE 2: Eadie-Hofstee plots of ATPase kinetic data obtained in the presence and absence of dinitrophenol. Incubations were performed in 0.5 M sucrose, 50 mM Tris-sulfate, 10 mM $MgSO_4$, 5 mM K_2SO_4, 4 mM PEP, 0.04 mg mL^{-1} of submitochondrial particle protein, in medium containing about 0.7 atom % excess $H_2{}^{18}O$. Incubations were from 1 to 30 min, pH 7.5, 25 °C, and dinitrophenol was 0.5 mM where added.

sponses were analyzed, namely, change in ATPase activity and in O:P ratio. The increase in velocity upon addition of dinitrophenol was highly significant as shown by the paired sample t test ($P = 0.004$), whereas the increase in O:P ratio was not significant ($P = 0.726$). Therefore, dinitrophenol increased the ATPase activity without changing the O:P ratio.

The general two-site model (sites interacting or noninteracting; Figure 1) requires that the values of the fitted constant, "c" (eq 7), not be significantly different from zero, and indeed the 95% confidence interval for "c" included zero in each of the four cases. The data were therefore refitted to an equation identical with eq 7 but without the constant. The results are shown in Table VII. The best-fitting values for n for the independent catalytic site model tend to lie just above the upper limits of the mechanistically realistic value of 4, and, if two independent sites are actually operative, it would appear that one site catalyzes no exchange and the other complete exchange. This is in contrast to the negative cooperative model where there appeared to be a low and a high exchanging site.

Discussion

Intermediate Mitochondrial P_i-$H_2{}^{18}O$ Exchange. The results presented in this paper extend the previous finding on the relative insensitivity of P_i-$H_2{}^{18}O$ exchange to inhibition by As_i, provided that an ATP-regenerating system is included to prevent ADP accumulation (DeMaster and Mitchell, 1973; Mitchell et al., 1975b) and provide direct evidence for the involvement of an intermediate type of P_i-$H_2{}^{18}O$ exchange accompanying ATP hydrolysis. The intermediate exchange appears to be closely associated with the ATP synthetase complex, as judged by the sensitivity of ATPase activity to oligomycin, and the fashion in which dinitrophenol stimulates ATPase without significantly altering the O:P ratio observed for a fixed ATP concentration. The presence of this exchange accounts for the well-known resistance of P_i-$H_2{}^{18}O$ exchange to dinitrophenol, and indeed when the elevation of ATPase by uncoupler is considered, serves to increase the rate of exchange from H_2O into the medium P_i (Mitchell et al., 1975a). The intermediate exchange also doubtless acounts for the high rates of oxygen exchange (ATP-$H_2{}^{18}O$ and P_i-$H_2{}^{18}O$) measured relative to P_i-ATP exchange (Boyer, 1967; Mitchell et al., 1967) since it allows for an incorporation of oxygen into P_i by a process associated with ATP hydrolysis rather than with P_i-ATP exchange. It is suggested that hydrolysis of ATP occurs at sites on F_1 in such a fashion as to produce an intermediate P_i-$H_2{}^{18}O$ exchange, thereby enriching enzyme-bound P_i derived from ATP rather than medium P_i. The elevation of the O:P ratio as ATP concentration decreased proved to be invaluable in demonstrating the intermediate character of the exchange proceeding in the presence of an ATP-regenerating system, since it predicted that relatively large losses of ^{18}O from medium P_i would have to occur if a medium exchange were to be implicated. The insensitivity of the $P_i = H_2{}^{18}O$ exchange reaction to dinitrophenol is compatible with the idea that the action of dinitrophenol does not directly involve the terminal step of the transphosphorylation reaction.

Evidence for Multiple Forms of Catalytic Sites Involved in ATP Hydrolysis. In general, curvature of Eadie-Hofstee plots of the type shown in Figure 2 is indicative of the presence of more than a single form of catalytic site engaged in catalysis (Cleland, 1970; Segel, 1975). It is proposed that the change in O:P ratio as the ATP concentration was altered is likewise indicative of the presence of more than one form of catalytic site engaged in the hydrolysis reaction. This behavior could occur, for example, if there were two types of ATPase such that the amount of ATP hydrolyzed by one form represented quite a different fraction of the total ATP hydrolyzed, depending on the particular substrate concentration used. Provided that the ATPases had a different capacity for catalyzing an intermediate exchange, the O:P ratio would be expected to change with change in ATP concentration. This argument can be extended to cases where more than two types of catalytic site are present, and is not restricted to independent sites. Thus, the increase in O:P ratio as the ATP concentration was decreased indicates the presence of a site with a high affinity for ATP with a high-exchanging capacity, and one or more lower affinity sites with a lower exchange capacity. Other workers have suggested the existence of multiple forms of hydrolytic sites associated with mitochondrial ATP. Schuster et al. (1975) have proposed that site-site interactions on mitochondrial ATPase gave rise to negative cooperative behavior associated with ATP hydrolysis. Adolfsen and Moudrianakis (1976) have provided evidence for polymorphic forms of ATPase. The oxygen exchange data presented in this paper provides a new type of evidence for heterogeneity of catalytic site involved in ATP hydrolysis in submitochondrial vesicles. Additionally, the exchange reaction appears to be a more sensitive method for detecting this heterogeneity under some conditions than that based on analysis of ATPase data, as can be seen in the case where dinitrophenol was present. Here, the curvature of the Eadie-Hofstee plot was not very pronounced; yet the observed ratio varied from about 1.3 to 3, over the concentration range of substrate used. This of course illustrates a limitation of the use of kinetic data from initial rate studies to detect the possible existence of multiple enzyme or catalytic site forms, since the resultant curvature of replotted data may not show much curvature even though more than one form of enzyme is present (Segel, 1975).

If the discussion is limited to cases involving only two forms of ATPase, the possible cases include: (1) noninteracting sites (e.g., dimorphic enzyme forms); (2) interacting sites (negatively cooperative interactions); and (3) a single catalytic site that is modified by occupation of a second ATP at an allosteric site. The last case may be considered as a special case[4] of case 2, and neither the kinetic nor the exchange data will help to distinguish it from case 2. Means of further examining cases 1 and 2 are discussed below.

Evaluation of Possible Models Involving Multiple Forms of Catalytic Sites. As discussed previously, experiments with inhibitors indicated that the exchange capacity associated with ATPase activity was mitochondrial in origin. The studies did not, however, enable a distinction to be made between the existence of multiple forms of ATPase, e.g., polymorphic variants, and the case where there existed a single form of ATPase which possessed multiple nucleotide binding sites, such that progressive binding of ATP altered the properties of one or more catalytic sites. The interpretation of the kinetic data is also complicated by the existence of a coupling membrane, since the vesicle might then be expected to behave as a supramolecular complex. One purpose for the inclusion of dinitrophenol in some of the experiments was to attempt to minimize interactions between ATPase molecules in the same vesicle. Such interactions might be expected to occur as a consequence of some form of transmembrane potential associated with ATP hydrolysis and the inclusion of uncoupler might be expected to abolish interactions depending upon the energization of the membrane.

The simplest types of models and indeed the only types that can be evaluated from the present data are those based on only two types of catalytic site. One object of the work was to see if the data were compatible with these relatively simple model types. As discussed below, the data were compatible with this simple system, although this does not prove, of course, that only two forms of catalytic site are involved. It does mean that only two forms of catalytic site need be postulated to account for the kinetic and the exchange data. A second object was to see if a distinction could be made between different types of molecules involving two catalytic sites. Formally, this problem is similar to making a distinction between the case where two independent enzymes are present and catalyzing a common reaction, and the case where a single enzyme displaying negative cooperativity is catalyzing the reaction. The term negative cooperativity is used here in its commonly accepted sense to denote that the occupancy of a catalytic site by the first ATP bound produces some change in the enzyme, such that the apparent K_m for the second ATP bound is increased.[5]

Since the approach used to make the distinction between the interacting and the noninteracting site models is novel and is based on making simultaneous measurements of initial rates and intermediate exchange, the rationale for the procedure will be discussed briefly.

The kinetic constants obtained from the analysis of initial rate data were used to calculate the velocities attributed to each of the two enzyme forms for a given ATP concentration. For the interacting site model, the velocity attributed to the high affinity site must first increase, as the substrate concentration increases from zero, and then the velocity must begin to decrease as the substrate concentration increases further, since there will be a progressive conversion of the singly occupied enzyme into the doubly occupied form. In contrast, for the independent site model, the velocity associated with the high affinity site must increase continually with increase in ATP concentration, and this value must approach its limiting value as the substrate concentration becomes saturating. In this case, both enzyme forms contribute to the observed total velocity as the substrate concentration becomes very large. Similar inferences may be made about the effect of lowering the substrate concentration. In general, the proportion of the total velocity attributed to the high and the low affinity sites will be different for the two different models for each ATP concentration considered. If the high and low affinity sites are characterized by a capacity for intermediate exchange unique to each, i.e., each site has a characteristic O:P ratio, then the estimates of this ratio (based on observed ratios which must be composites reflecting the intrinsic ratios and the relative amounts of ATP hydrolyzed by each site) must differ for the two models. For O:P ratios calculated in this way to have physical significance, in terms of an intermediate exchange involving P_i, the values of the intrinsic ratios must lie between 1 and 4, since a single oxygen must be incorporated due to hydrolysis, and the maximum incorporation cannot exceed the maximum number of oxygen atoms in a P_i molecule. As shown in Tabel VII, this was not found to be the case for the noninteracting site model, and the discrepancy was most marked in the case of n_1, with dinitrophenol present. The value obtained (5.4) exceeds 4 by more than twice the standard error. On the other hand, the value calculated for the corresponding site for the interacting site model (i.e., 2.66) was within the mechanistically feasible limits. As shown in the Appendix, values for n_1 and n_2 for the negative cooperativity model must lie between the values calculated for the independent site model.

Expressed in other terms, the results presented in Table VII show that the way in which the O:P ratio changed with change in ATP concentration was so pronounced that, if two separate ATPases of the type indicated by the Eadie–Hofstee plots were to be responsible, the high affinity enzyme must incorporate four oxygen atoms from water into each P_i released, whereas the low affinity enzyme must incorporate only one oxygen from water. This possibility can be tested directly by carrying out the enzyme-catalyzed hydrolysis of ATP in very highly enriched $H_2{}^{18}O$, and measuring the multiplicity of labeling in the product directly, using a P_i derivative suitable for direct mass spectrometric analysis. Results from this type of experiment show that the O:P ratios measured in the usual fashion are not composites of singly and quadruply labeled P_i species (Mitchell, R. A., unpublished results).

Consequently, for two-catalytic site models, the interacting site model is to be preferred over the noninteracting site model. Various authors (see Kayalar et al., 1977, and references therein) have suggested that there may be site–site interactions on a common ATPase molecule, and such suggestions would be compatible with the type of behavior described here. However, it should be noted that the occurrence of three or more different forms of independent site might occur, and be responsible for the effects of ATP concentration on the kinetic

[4] The case where a single catalytic site interacts with a single regulatory site has been considered by Harper (1973) to be a special case of the two catalytic site model, and, although Schuster et al. (1975) have favored the catalytic/regulatory site model to explain their kinetic data, these two alternatives cannot be distinguished readily. In the present analysis this would imply the existence of a catalytic site subjected to ATP control via a regulatory site, such that as the catalytic activity (ATP hydrolysis) was modified by occupancy of the regulatory site, the intrinsic O:P ratio was decreased. The distinction between these possibilities may require the application of both kinetic and nucleotide binding studies, e.g., photoaffinity labeling, to study the topography of F_1.

[5] It should be noted that this definition does not preclude the possibility that the modified enzyme may have a greater turnover of substrate than the unmodified, so that at high substrate concentrations the catalytic capacity of the enzyme is increased, rather than impaired, as the term negative cooperativity might seem to imply.

and exchange behavior.

The observation that the intermediate exchange is resistant to uncouplers is of importance since it suggests that a coupling membrane is not needed for this exchange to occur. This points to the possibility of using simpler ATPase preparations to study the exchange reaction. Preliminary studies on the Triton-solubilized mitochondrial ATPase preparation (Linnett et al., 1975) showed that this enzyme also catalyzed an exchange of oxygen from water into P_i during ATP hydrolysis, in such a manner that the exchange was elevated as the ATP was decreased (Lamos, C. M., unpublished results). This preparation may be more amenable to study than the particulate ATPase.

The mechanism giving rise to intermediate exchange accompanying ATP hydrolysis catalyzed by mitochondrial ATPase is not known. Possibly it resembles the myosin-catalyzed exchange (Bagshaw et al., 1975) and reflects the dynamic reversal of ATP synthesis and hydrolysis on the enzyme surface, as has been proposed by Rosing et al. (1977). There is growing evidence to suggest that the equilibrium of enzyme-bound intermediates associated with various phosphotransferases may be quite different from the position of equilibrium of the same reactants and products in solution (Bagshaw and Trentham, 1973; Danneberg and Cleland, 1975). Similar information would clearly be relevant to the study of the terminal steps in oxidative phosphorylation. The occurrence of an uncoupler-resistant intermediate exchange may prove useful in this respect, particularly if it can be shown that the exchange is related to the hydrolysis and reesterification of ATP on the enzyme surface.

Acknowledgments

The authors are grateful to Larry Witt for his able technical assistance.

Appendix

The independent and the interacting site models both predict that the O:P ratios will approach limiting values as the ATP concentration approaches zero or infinity. For the independent site model, it follows from eq 1 that:

$$\lim_{S \to 0} \text{O:P} = \frac{n_1 V_{m(l)} K_{m(h)} + n_2 V_{m(h)} K_{m(l)}}{V_{m(l)} K_{m(h)} + V_{m(h)} K_{m(l)}}$$

and

$$\lim_{S \to \infty} \text{O:P} = \frac{n_1 V_{m(l)} + n_2 V_{m(h)}}{V_{m(l)} + V_{m(h)}}$$

For the interacting site model, it follows from eq 2 that:

$$\lim_{S \to 0} \text{O:P} = n_1$$

and

$$\lim_{S \to \infty} \text{O:P} = n_2$$

This means that, for the interacting site model, the limiting values for the O:P ratio are in fact the intrinsic values for each site. For the independent site model, the limiting values as [ATP] approaches infinity are a composite value, resulting from a site with an intrinsic O:P greater than the limiting value and a site with and O:P ratio less than the limiting value. That is, the intrinsic O:P for the low exchanging site (characterized by n_2) must be numerically smaller for the independent site model than the corresponding value for the interacting site model. Similar reasoning for the limiting O:P ratio as [ATP] approach zero shows that the intrinsic O:P ratio for the independent site characterized by n_1 must be larger than the corresponding value for the dependent site model.

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