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# MITOCHONDRIAL-CATALYZED ATP HYDROLYSIS IN HIGHLY ENRICHED [$^{18}$O]$H_2O$

## FREQUENCY DISTRIBUTIONS OF $^{18}$O-LABELLED $P_i$ SPECIES

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## Summary

1. The 2,4-dinitrophenol-resistant intermediate phosphate ⇌ water oxygen exchange reaction which accompanies the hydrolysis of ATP catalyzed by heart submitochondrial vesicles was studied in highly enriched $^{18}$O-labelled water. The phosphate formed was converted into the tris(trimethylsilyl) phosphate derivative which was analyzed by chemical ionization mass spectrometry. The amount of exchange relative to that of ATP hydrolysis, and the ATP dependence of the exchange on the ATP concentration were measured. The results were in good agreement with previous studies carried out on water of low enrichment. However, the new procedure enabled the determination of the relative frequency distributions of phosphate species with 1, 2, 3, or 4 oxygen atoms derived from water. Oxygen from water was not found only in singly or quadruply $^{18}$O-labelled species. This finding further helps to exclude the possibility that the vesicles contain two separate ATPases, one non-exchanging, the other highly exchanging.

2. The frequency distributions of $^{18}$O-labelled $P_i$ species were measured at high (6 mM) and low (0.01 mM) ATP, as well as at three intermediate ATP concentrations. Regression analysis was carried out to see if the frequency distributions found with the intermediate ATP concentrations were simple composites of the patterns obtained at the high and low ATP levels. The analysis did not support the idea that the phosphate formed at the intermediate ATP levels was a binary mixture of phosphate produced by the hydrolysis of ATP at a high and a low affinity ATP site. This finding excluded models involving two independent Michaelis-Menten ATPases, as well as simple two-site negatively cooperative models.

3. Oxygen from water was incorporated into all four oxygens of phosphate, and the extra oxygen (i.e., that incorporated over and above the single oxygen needed for the hydrolytic step) was distributed approximately binomially. This implies that the four oxygen atoms of $P_i$ were equivalent during the time enzyme-bound $P_i$ underwent exchange. Thus a pseudorotation mechanism in which one oxygen was strongly liganded to a group on the enzyme does not appear likely. The exchange may thus reflect the dynamic reaction, ATP + $H_2O \rightleftharpoons$ ADP + $P_i$ on the enzyme surface. The effect of ATP concentration upon exchange may thus reflect the extent of hydrolysis and reesterification which occurs prior to $P_i$ release from the enzyme.

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## Introduction

In the presence of an ATP-regenerating system, the $P_i \rightleftharpoons$ ATP and ATP$\rightleftharpoons H_2O$ exchanges catalyzed by submitochondrial vesicles are suppressed [1,2] and only a $P_i \rightleftharpoons H_2O$ exchange activity accompanies ATP hydrolysis. An early study indicated that this residual exchange was inhibited by inorganic arsenate, indicating that medium $P_i$ was the major reactant [3]. However, later studies showed that provided a large excess of the ATP-regenerating enzyme was present, neither phosphate nor inorganic arsenate inhibited ATP-driven energy-linked reduction [4], thereby suggesting that these compounds failed to bind to the catalytic site in the absence of ADP. Subsequent experiments showed that in the absence of ADP the $P_i \rightleftharpoons H_2O$ exchange was not inhibited by inorganic arsenate or dinitrophenol, did not involve medium $P_i$, and was elevated relative to the rate of hydrolysis as the ATP concentration was decreased [5].

The exchange is of special interest in energy-transduction studies because it affords an opportunity to study the chemistry of the steps involved in making or breaking the pyrophosphate bond of ATP. Since the exchange is resistant to 2,4-dinitrophenol, this uncoupler may be used to abolish reactions which may arise as a consequence of the energization of the vesicle as a whole, e.g., interactions between ATPase units in the vesicle, mediated by a coupling membrane.

The present work was undertaken to measure the actual frequency distributions of $P_i$ species (derived by ATP hydrolysis) containing 1, 2, 3, or 4 oxygen atoms from water per $P_i$ molecule. These data can provide information about the equivalence of oxygen atoms in enzyme-bound $P_i$ and may serve to indicate what if any restrictions are imposed upon the movement of $P_i$ at the catalytic site. The possibility was also considered that the analysis of frequency distributions of multiply-labelled $P_i$ species at different ATP concentrations, might also allow the intermediate exchange to be used to estimate the partial velocities of ATP hydrolysis at each of two hypothetical sites, namely high and low affinity sites.

To the extent that each site imprints on phosphate released a distinctive pattern of oxygen exchange, it is possible to resolve the distribution patterns of $^{18}O$ in phosphate, to see if they are composites of binary phosphate fluxes from two sites. If so, the relative proportions of the binary components should corresponds to the relative proportions of phosphate released from each site. A preliminary report of the present study has been published [6].

## Methods

Submitochondrial vesicles were prepared just before use by the ultrasonic disruption of frozen bovine heart mitochondria. Incubation mixtures for experiments using high enrichment water (about 90 atom% $^{18}O$) were prepared by lyophilizing small aliquots (usually 0.5 ml) of a stock solution (pH 7.5) containing 0.25 M sucrose, 50 mM Tris sulfate, 10 mM $K_2SO_4$. 10 mM $MgSO_4$, 4 mM phospho*enol*pyruvate, and 0.5 mM 2,4-dinitrophenol. Final traces of water were removed by addition of absolute ethanol to the residue. Ethanol was removed at reduced pressure, and the treatment repeated two or three times with about 3 ml of ethanol, to yield a dry residue which was stored in a desiccator until needed.

Reaction mixtures were reconstituted by addition of the appropriate amount of $^{18}O$-enriched $H_2O$ (Miles Laboratory, Elkhart, IN, or Prochem, Maplewood, NJ) and lyophilized pyruvate kinase (Type III, Sigma Chemical Co., St. Louis, MO). Freshly-prepared submitochondrial vesicles were added in a small volume (10 $\mu$l) and the suspension incubated with gentle shaking at 25°C for 5 min, at which time the reaction was started by the addition of ATP (also in a small volume). Samples were incubated for various lengths of time (5 to 40 min, depending upon the ATP concentration), to yield approx. 1 to 2 mM $P_i$ from ATP hydrolysis. The tubes were closed with a high-vacuum stop-cock fitted to a ground glass joint. The reaction was stopped by plunging the end of the tube into liquid nitrogen, followed by recovery of the enriched water by distillation under reduced pressure (the received was cooled in liquid nitrogen and the reaction mixture kept at −15°C by immersion in an ice-salt mixture). Perchloric acid (0.5 N) was added to the reaction mixture residue, and the $P_i$ was purified by way of the triethylamine molybdate and the magnesium ammonium phosphate complexes, followed by conversion of the latter into phosphoric acid using a Dowex 50 $H^+$ column [7]. To the column eluant (about 5 ml) was added 1 drop of pyridine and the solution was taken to dryness under reduced pressure using $CaCl_2$ as desiccant. The pyridinium phosphate was dried overnight in a vacuum oven at 25°C and the dry salt was heated at 70°C for 1 h in a sealed reaction vessel with the silylation reagent, *N,O*-bis(trimethylsilyl)acetamide (BSA Ampul Kit, Supelco, Inc., Bellefonte, PA), using 0.1 ml of reagent per $\mu$mol of $P_i$. The formation of product was checked by gas chromatography.

*Mass spectrometric analysis*

The $^{18}O$ content was measured either as $CO_2$ or as the silylphosphate derivative. For the former, the guanidine HCl method was used to incorporate oxygen from $H_2O$ or $P_i$ into $CO_2$. The $CO_2$ was collected in an evacuated bulb [7]. A CEC 21-401 mass spectrometer was used to measure the relative intensities of peaks $m/e$ = 44, 46, and 48 (corresponding to 0, 1, and 2 $^{18}O$ per $CO_2$, respectively). This method was used routinely to analyze the $^{18}O$ content of the incubation water before and after the hydrolysis was carried out. In contrast, tris(trimethylsilyl) phosphate was analyzed with a chemical ionization spectrometer [8], using the 315, 317. . .323 cluster.

*Corrections for Si isotopes*

Corrections for spillover of $^{29}Si$ and $^{30}Si$ into adjacent $^{18}O$-containing peaks were made in a stepwise fashion, as described by Eargle et al. [8], except that the corrections were calculated on the basis of the binomial equation. Details of a similar method have been published independently by Boyer et al. [9].

*Interpretation of mass spectral data*

Frequency distributions were calculated from peak heights using a programmable calculator. Binomial distributions were calculated using a form of the equation given by Matsumoto and Hammes [10]. The equation was used for three purposes. These were: to correct for the occurrence of $^{29}Si$ and $^{30}Si$ in the silyl phosphate derivative; to adjust the measured $[^{18}O]/P_i$ frequency distributions obtained in 90 atom% $[^{18}O]H_2O$ to the theoretical values for 100 atom% $[^{18}O]H_2O$; to compare various observed frequency distributions with those which would be expected for binomially-distributed label. Observed and predicted frequencies were compared using a likelihood ratio test ($G$ test) for goodness of fit [11]. Determination of the composition of test mixtures from their mass spectral data was carried out by regression analysis using an IBM 360/67 computer and a statistical analysis program (Constat) furnished by Wayne State University Computing Center.

*Regression analysis of actual and suspected binary mixtures*

Let the frequency distributions of $[^{18}O]/P_i$ for the component which has the low $[^{18}O]$ content be $f(i)$low (where $i = 0$, 1, 2, 3, and 4 for synthetic $P_i$ mixtures, and 1, 2, 3, and 4 for $P_i$ formed by hydrolysis). Let $f(i)$high represent the corresponding distributions for the component of the mixture with the higher $^{18}O$ content, and let the distributions for the mixture be represented by $f(i)$-mix. The frequency distributions for the individual components are related to those of the mixture by the equation:

$$p \times f(i)\text{high} + q \times f(i)\text{low} + c = f(i)\text{mix}$$

where $p$ and $q$ are the fractional components of the high and low enriched phosphate species, and $c$ is a fitting constant. Since there are 5 (4 in the case of $P_i$ formed by hydrolysis of ATP) linear simultaneous equations which can be obtained from each mixture, and three unknowns ($p$, $q$, and $c$), the equations can be solved by standard regression analysis techniques. Constant '$c$' may be viewed as a fitting parameter which has no physical significance for the binary model. For a precise fit to the model, the constant should not differ significantly from zero. Non-zero values may be encountered if there is a systematic error in the analysis of if the model is incorrect. The size of '$c$' in such cases will determine how closely the sum of $p$ and $q$ is to 1, the theoretical sum for a binary mixture.

## Results

*Validation of methodology for calculating frequency distributions*

A highly-enriched $P_i$ sample (Miles Laboratories, '85.1 atom%') was estimated to contain 84.9 atom% $^{18}O$ (average of 3 samples) by the guanidine HCl

TABLE I

OBSERVED AND CALCULATED FREQUENCY DISTRIBUTIONS AND $^{18}O$ CONTENT OF THREE BINARY MIXTURES CONTAINING ENRICHED AND NONENRICHED PHOSPHATE

Stock solutions of 78.0 and 0.204 atom % $^{18}O$-labelled phosphate were mixed in known proportions to give three binary mixtures containing the calculated enrichments shown. Mixtures and both components were converted into tris(trimethylsilyl) phosphate. The latter was analyzed by mass spectrometry.

| Mixture | | $f_0$ | $f_1$ | $f_2$ | $f_3$ | $f_4$ | Atom % | $G$ |
|---|---|---|---|---|---|---|---|---|
| 1 | Obs. | 0.772 | 0.013 | 0.036 | 0.088 | 0.090 | 17.8 | 0.005 |
| | Cal. | 0.771 | 0.006 | 0.033 | 0.018 | 0.001 | 18.5 | |
| 2 | Obs. | 0.547 | 0.017 | 0.071 | 0.183 | 0.181 | 35.8 | 0.001 |
| | Calc. | 0.532 | 0.013 | 0.068 | 0.190 | 0.197 | 37.7 | |
| 3 | Obs. | 0.277 | 0.029 | 0.144 | 0.290 | 0.261 | 55.7 | 0.027 |
| | Calc. | 0.287 | 0.019 | 0.103 | 0.290 | 0.301 | 57.5 | |

method, and 85.4 atom% $^{18}O$ by the silylation method, after correcting for $^{29}Si$ and $^{30}Si$ spillover.

Three mixtures were prepared containing non-enriched and 78.0 atom% $^{18}O$-labelled $P_i$ in different proportions. Table I shows the corrected and normalized frequency distributions of $P_i$ species containing 0, 1, 2, 3, or 4 $^{18}O$ per $P_i$. The observed frequency distributions of the $^{18}O$-labelled phosphate species in the binary mixtures can be used to estimate the composition of the mixtures by regression analysis, provided that the frequency distributions for the individual components are known. The results are summarized in the first part of Table III. The value of $c$ was significantly greater than zero, which indicated a small systematic error in the analysis. Nevertheless, the estimates for the fractional composition of the mixtures were close to the expected values, thus confirming the applicability of the method for binary mixture analysis.

TABLE II

FREQUENCY DISTRIBUTION OF $^{18}O$-CONTAINING PHOSPHATE SPECIES DERIVED BY ATP HYDROLYSIS IN $[^{18}O]H_2O$

Incubation mixtures (pH 7.5, 25°C) contained 0.25 M sucrose, 50 mM Tris sulfate, 10 mM $MgSO_4$, 5 mM $K_2SO_4$, 4 mM phospho*enol*pyruvate, 0.5 mM dinitrophenol, and 0.6 mg pyruvate kinase, and 1.15 mg of mitochondrial protein/ml, in a final volume of 0.3 ml 90.1 atom % $[^{18}O]H_2O$. Reactions were started by addition of ATP 5 min after addition of submitochondrial vesicles, to give a final concentration of 6, 0.2, 0.1, 0.05, or 0.01 mM ATP. Respective incubation times were 1, 5, 10, 30 and 60 min. ATP and vesicles were each added in 10 µl aliquots.

| ATP (mM) | | $f_1$ | $f_2$ | $f_3$ | $f_4$ | O:P | $G$ |
|---|---|---|---|---|---|---|---|
| 6.00 | Obs. | 0.686 | 0.220 | 0.059 | 0.036 | 1.44 | 0.159 |
| | Calc. | 0.621 | 0.320 | 0.055 | 0.003 | | |
| 0.20 | Obs. | 0.562 | 0.286 | 0.107 | 0.046 | 1.63 | 0.120 |
| | Calc. | 0.493 | 0.393 | 0.105 | 0.009 | | |
| 0.10 | Obs. | 0.521 | 0.303 | 0.123 | 0.055 | 1.71 | 0.124 |
| | Calc. | 0.445 | 0.414 | 0.128 | 0.013 | | |
| 0.05 | Obs. | 0.446 | 0.297 | 0.182 | 0.074 | 1.88 | 0.137 |
| | Calc. | 0.353 | 0.439 | 0.182 | 0.025 | | |
| 0.01 | Obs. | 0.186 | 0.272 | 0.277 | 0.264 | 2.62 | 0.184 |
| | Calc. | 0.097 | 0.343 | 0.402 | 0.157 | | |

*ATP hydrolysis in [$^{18}O$]$H_2O$*

The pattern of $^{18}O$ incorporation into $P_i$ formed by ATP hydrolysis (using 5 different ATP concentrations) was examined next. Table II shows the relative frequency distributions of $P_i$ species containing 1, 2, 3, or 4 oxygen atoms derived from water. Corrections were made to the measured peak intensities to correct for $^{29}Si$ and $^{30}Si$ spillover and to adjust the peak heights to the values which would be obtained in 100 atom% [$^{18}O$]$H_2O$. The increase in O : P ratio as the ATP concentration was decreased is in good agreement with previous values obtained in similar experiments but in water of low $^{18}O$ enrichment [5], At 6 mM ATP, singly and doubly $^{18}O$-labelled species accounted for about 90% of the total $P_i$ species. As the ATP level was decreased from 6 to 0.01 mM, the amount of singly labelled species was decreased from approx. 70 to 20%. The appearance of about 25% quadruply labelled species at the lowest ATP level tested showed that all four oxygen atoms of $P_i$ were able to exchange with oxygen from water.

Table II also shows the theoretical distributions for a given $^{18}O$ content, calculated on the assumption that the extra oxygen (i.e. that which has been incorporated over and above the single oxygen atom needed for hydrolysis) is binomially distributed. Agreement between observed and calculated frequencies is reasonably good. The *G*-statistic values for the five sets of data showed no marked trend either toward or away from a binomial distribution of extra oxygen from water in $P_i$, as the ATP concentration was reduced.

If the distribution patterns observed with the extreme ATP concentrations

TABLE III

RESOLUTION OF ACTUAL OR SUSPECTED BINARY PHOSPHATE MIXTURES INTO THEIR INDIVIDUAL COMPONENTS BY REGRESSION ANALYSIS OF THE $^{18}O$ FREQUENCY DISTRIBUTIONS

Mixtures of highly-enriched and non-enriched phosphate were prepared from solutions of the two types of phosphate. The other 5 samples were obtained by enzyme-catalyzed ATP hydrolysis. *p* represents the component with the higher $^{18}O$ content (enriched phosphate in the case of binary mixtures, or phosphate released from a high-exchanging site, in the case of ATP hydrolysis). Expected values are based on the composition of mixtures as prepared, or on the partial velocities attributed for a negative cooperativity model, using the reported kinetic constants [5]. In the case of $P_i$ formed by ATP hydrolysis, the expected fractional composition of a mixture at a particular ATP concentration was calculated for a two-site negative cooperativity model (Equation 2) using the values, $K_s$ = 0.11 mM; $V$ = 0.24 mM · $min^{-1}$; alpha = 2.54; beta = 3.023. These values are reported by Russo et al. [5], except for $V$ which was erroneously reported as 0.21.

| Source of sample | Fractional composition | | | | |
|---|---|---|---|---|---|
| | By regression | | | Expected | |
| | *p* | *q* | *c* | *p* | *q* |
| Mixture 1 | 0.203 | 0.758 | 0.008 | 0.237 | 0.763 |
| Mixture 2 | 0.436 | 0.526 | 0.006 | 0.483 | 0.517 |
| Mixture 3 | 0.617 | 0.226 | 0.032 | 0.737 | 0.263 |
| 6 mM ATP | | (*q* set at 1.000) | | 0.015 | 0.985 |
| 0.2 mM ATP | 3.079 | 1.160 | −0.810 | 0.316 | 0.684 |
| 0.1 mM ATP | 3.769 | 1.164 | −0.983 | 0.481 | 0.519 |
| 0.05 mM ATP | 4.134 | 1.037 | −1.042 | 0.651 | 0.349 |
| 0.01 mM ATP | | (*p* set at 1.000) | | 0.902 | 0.098 |

are accurate representations of the limiting patterns obtained as the ATP concentration approaches either saturating levels, or zero concentration, and if the intermediate ATP levels produce distributions which are composites of these two extremes, then it should be possible to estimate the composition of the intermediate levels in terms of the relative proportions of the high and low ATP components. Such an analysis was tried, using the same procedure that was used for the regression analysis of binary $P_i$ mixtures. The analysis provided no evidence to support the idea that the frequency distributions observed with the three intermediate ATP levels were in fact composites of this type (Table III). This is because (1), the constant term in the fitted equation was significantly different from zero in all three cases, and (2), the ratio of the two variables which serve to estimate the relative rates of flux through the two pathways did not change with ATP concentration in a way which was consistent with this simple model, and (3), the fitted values of the two coefficients were too large to accommodate the model. The two coefficients represent the fractions of $P_i$ which are attributed to each flux, and for an exact fit, the coefficients for the two fluxes should sum to unity. In all three cases the sum of the fitted coefficients was greater than 4.

## Discussion

### *The mechanism of the exchange reaction*

Two viewpoints have been advanced to account for the mitochondrial oxygen exchange reactions. Korman and McLick [12] proposed that the synthesis or hydrolysis of ATP involves the formation of a stable pentacovalent intermediate of phosphorus, and that pseudorotation of this compound occurred, thereby permitting an exchange of apical and equatorial oxygen atoms and allowing for an exchange of water oxygen into the intermediate. This mechanism was patterned after that proposed for the hydrolysis of strained cyclic phosphodiesters, where there is good evidence to support pseudorotation [13]. Kozlov and Skulachev [14] also consider that mitochondrial oxygen exchange occurs by way of a pentacovalent phosphorus intermediate. Mitochondrial exchange studies carried out using analogues of ATP not susceptible to hydrolysis by mitochondrial ATPase did not invalidate the Korman and McLick hypothesis (Holland et al. [15]). However, Boyer and coworkers [2,16] have suggested that the exchanges represent the dynamic reaction, $ATP + H_2O \rightleftharpoons ADP + P_i$, as it occurs on the enzyme surface. This type of reversal appears to account for the myosin-catalyzed $P_i \rightleftharpoons H_2O$ exchange which accompanies ATP hydrolysis in the presence of Mg [17,18], and also the $ATP \rightleftharpoons H_2O$ oxygen exchange reaction catalyzed by chloroplasts when exposed to light [19].

A new approach to the study of the mitochondrial oxygen exchange reactions was made possible by the discovery that submitochondrial vesicles catalyzed an intermediate $P_i \rightleftharpoons H_2O$ exchange which was not inhibited by dinitrophenol, and which was increased, relative to the rate of ATP hydrolysis as the concentration of ATP was decreased [5]. These observations pointed to the value of measuring actual frequency distributions of $^{18}O$-containing $P_i$ as a means of gaining further information about possible site-site interactions.

*The effect of ATP concentration upon exchange*

From an analysis of the kinetics of ATP hydrolysis and the intermediate exchange as a function of ATP concentration, it was concluded that in the presence of dinitrophenol two independent sites were not involved in ATP hydrolysis. The exchange and kinetic data can be explained in terms of two independent sites only if one site catalyses exchange with all four oxygens of $P_i$ with water oxygen, and the other site catalyzes no exchange. This pattern was not observed in the present series of experiments and this finding is in agreement with the earlier conclusion that the alteration in exchange rate with ATP concentration was not due to two independent types of Michaelis-Menten ATPase. The next simple model considered was a two-site negative cooperativity model. The term negative cooperativity is used here in the conventional kinetic sense to denote that the binding of substrate to the first site produces an E · ATP complex with a larger $K_m$ for the second molecule of substrate. At low substrate concentrations, the flux will be predominantly through the symmetrical E · ATP and ATP · E complexes, whereas at saturating levels of substrate the flux will be through the ATP · E · ATP complex. This model is not identical to a flip-flop model, where binding of substrate at the second site promotes catalysis and/or release of product from the first site. The negative cooperative model can be tested by examining the frequency distributions of phosphate species (derived from ATP by hydrolysis in highly enriched [$^{18}$O]-$H_2O$) containing 1, 2, 3, or 4 oxygen from water per $P_i$. This approach depends on using ATP concentrations which are sufficiently high or low to permit the measurement of the characteristic frequency distributions associated with the release of $P_i$ from doubly- or singly-occupied sites. The frequency distributions at intermediate ATP concentrations can then be evaluated by regression analysis, to see if they are composites made up of the extremes in differing proportions (heterogeneous binary fluxes). Analysis of these data (Table III) did not support the idea that the frequency distribution patterns obtained at intermediate ATP levels were composites of the limiting patterns. The lowest ATP concentration used was 1/10 the estimated $K_s$ of a singly occupied site, and the highest ATP level was about 20 times the estimated $K_s$ for a doubly occupied site (based on the kinetic constants for an interacting site model, dinitrophenol present, Russo et al. [5]). Thus, the selected ATP concentrations were appropriate for the model tested.

The exclusion of the two site negative cooperativity model means that other models must be considered to explain the ATP dependence of the intermediate exchange. Two features of the exchange should be noted. These are, (1), all four oxygen atoms in $P_i$ can be labelled with oxygen from $H_2O$, and (2), the extra oxygen appeared to be distributed binomially at all ATP levels used (homogeneous flux). This implies that all four oxygen were stereochemically equivalent during the steps involved in hydrolysis and exchange. In a conventional pseudorotation mechanism, at least one oxygen of the reactant undergoing hydrolysis would be expected to be strongly ligandeded to a group on the enzyme, in order to induce strain on the substrate. In studies with model compounds, it is the reduction in ring strain through the formation of a stable pentacovalent phosphorus intermediate which accounts for the high rates of exchange and hydrolysis associated with strained phosphodiesters [13]. These

results thus are in agreement with the suggestion by Boyer and coworkers [16] that the exchange most likely represents hydrolysis and formation of ATP on the enzyme surface, in a nonenergy-dependent reaction.

The resistance of the intermediate $P_i \rightleftharpoons H_2O$ oxygen exchange to dinitrophenol indicates that the effect of ATP on exchange most likely reflects site-site interactions. The suggestion by Kayalar et al. [20] for an alternating site mechanism was based on experiments on a coupled system which is not very well suited for such studies because the coupled vesicle shows strong negative cooperative effects with ATP hydrolysis. These effects seem to reflect interactions between ATPase assemblies mediated by the coupling membrane [5]. These interactions would be absent in the studies described here, because of the inclusion of uncoupler. A similar ATP concentration effect and a similar type of homogeneous flux with a soluble mitochondrial ATPase preparation [21] likewise point to site-site interactions between ATPase subunits as the cause of the ATP concentration effect on exchange. A combination of exchange studies and initial rate measurements appears to offer a useful approach to the study of site-site interactions between mitochondrial ATPase subunits, although the method has a general applicability.

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