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Relationships of Inosine Triphosphate and Bicarbonate Effects on F_1 ATPase to the Binding Change Mechanism

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Abstract

Two interesting previously reported properties of mitochondrial F_1 ATPase have been confirmed and have been examined by ^{18}O exchange measurements to assess if they are consistent with sequential participation of catalytic sites during ATP hydrolysis. These are the ability of HCO_3^- to increase reaction rate with apparent loss of cooperative interaction between subunits and the ability of ITP to accelerate the hydrolysis of a low concentration of ATP. The effect of HCO_3^- was tested at concentrations of ATP lower than previous measurements. The activation disappeared when ATP was reduced to 0.1 μM. The HCO_3^- activation at higher ATP concentrations did not change the extent of reversal of the cleavage of tightly bound ATP at the catalytic site, as measured by the average number of water oxygens incorporated with each P_i formed when 5 or 10 μM ATP is hydrolyzed. The data are consistent with sequential site participation with HCO_3^- acceleration of ADP departure after a binding change that stops ^{18}O exchange and loosens ADP binding.

When ITP concentration was lowered during net ITP hydrolysis by F_1 ATPase an increase in water oxygen incorporation into P_i formed is observed, as noted previously for ATP hydrolysis. The acceleration of the cleavage of a constant low concentration of [γ-^{18}O]ATP by concomitant hydrolysis of increasing concentrations of ITP was accompanied by a decrease in water oxygen incorporation with each P_i formed from the ATP. These results add to evidence for the binding change mechanism for F_1 ATPase with sequential participation of catalytic sites.

Key Words: F_1 ATPase; adenosine triphosphate; bicarbonate; ^{18}O exchanges.

Introduction

Recent studies in this laboratory have probed facets of a binding change mechanism for ATP hydrolysis by F_1 ATPases purified from mitochondria or

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chloroplasts (Gresser *et al.*, 1982; Kohlbrenner and Boyer, 1983; Boyer *et al.*, 1982). In this mechanism, ATP binds tightly at a catalytic site and is reversibly hydrolyzed to tightly bound P_i and ADP. The reversible cleavage of the bound ATP results in the incorporation of water oxygens into the bound P_i and ATP. At low ATP concentrations extensive incorporation of water oxygens into P_i released is observed. Increase in the ATP concentration results in the binding of ATP to another catalytic site on the multisubunit enzyme. Conformational changes accompanying the binding of this ATP loosen the binding of ADP and P_i and reduce the oxygen exchange. Although negative cooperativity of ATP binding occurs, the catalytic cooperativity between sites is such that substrate binding at one site greatly increases the rate of product release from another site. One goal of this laboratory is to continue to assess if a wide variety of observations made with the F_1 type ATPases are consistent with this alternating site mechanism. Studies are reported here with two previous observations whose consistency with sequential participation of catalytic sites[2] was unclear.

One observation that appeared to warrant further examination was the unusual effect of bicarbonate ions. Bicarbonate augments the initial velocity of ATP cleavage over a wide range of ATP concentrations and eliminates the nonlinearity of $1/v$ vs $1/S$ plots observed in the absence of bicarbonate (Ebel and Lardy, 1975). This nonlinearity was consistent with the binding of ATP at one site promoting the release of tightly bound ADP and P_i from another catalytic site. Such behavior made attractive the consideration that bicarbonate might act by promoting the binding change that loosens the tight binding of ADP and P_i and thereby terminating the oxygen exchange. We thus explored the effects of bicarbonate on the ATP modulation of oxygen exchange accompanying ATP hydrolysis. However, our results showed that bicarbonate does not change the extent of oxygen exchange at various ATP concentrations.

Another explanation for the bicarbonate effect needed consideration. At higher ATP concentrations, where the net ATP hydrolysis catalyzed by F_1 ATPase is rapid, only slightly greater than one water oxygen is incorporated into each P_i released. This means that the conformational change that loosens ADP and P_i binding and stops oxygen exchange is also rapid. In the model of Gresser *et al.* (1982) subsequent dissociation of this more loosely bound ADP can contribute significantly to rate limitation. Bicarbonate could aid this dissociation thereby increasing net reaction rate without changing the average number of water oxygens incorporated into each P_i formed. However,

[2]In previous publications the designation "alternating site participation" was used. Present evidence suggests that three identical catalytic sites may participate in sequence. The designation "sequential participation of catalytic sites" is preferable as alternating sites can be regarded as limited to only two catalytic sites.

bicarbonate was observed to accelerate ATP hydrolysis even at the lowest ATP concentration tested (40 μM). At sufficiently low ATP concentrations the binding change and not the dissociation of ADP was expected to be the major rate-limiting step. We thus explored the possibility that bicarbonate might lose its accelerating capacity when ATP concentrations are reduced below 1–2 μM. This was found to be the case. Therefore bicarbonate acceleration of ADP dissociation is a plausible explanation for its effects.

Other observations, whose possible consistency with the sequential participation of catalytic sites needed evaluation, were those of Schuster *et al.* (1976) on the effects of ITP on the hydrolysis of low concentrations of ATP. They observed an initial increase in the ATP hydrolysis rate as the ITP concentration was increased, followed by an inhibition of ATP hydrolysis at higher ITP concentrations. It appeared that these interesting findings could be explained by sequential site participation if at appropriate concentrations the binding of ITP could favor the release of tightly bound ADP and P_i from the enzyme, and medium ATP could successfully compete with ITP for empty catalytic sites. Were this the case, as increase in ITP concentration speeded up ATP hydrolysis, at a constant low ATP concentration, we would expect the extent of water oxygen incorporation into each P_i released to be decreased. Such effects of ITP are reported in this paper.

Experimental

Soluble F_1 ATPase was isolated from beef heart mitochondria according to Penefsky (1979). Protein concentrations were determined using the method of Bradford (1976) with Bio-Rad reagent and bovine serum albumin as a standard. IDP was purified on a Biogel P-10 column (1.0 × 25 cm) equilibrated with 0.05 M Tris-acetate, pH 7.6.

Enzymes used for the experiments were desalted by centrifugation through Sephadex G-50 columns (Penefsky, 1977) that had been equilibrated with 0.05 M Tris-acetate buffer, pH 7.6. Prior to passage through the centrifuge column F_1 ATPase was dissolved in 0.05 M Tris-sulfate buffer, pH 7.6, containing 1 mM EDTA (Leimgruber and Senior, 1976).

Measurements of P_i formed were carried out by continuous monitoring of the P_i release using a slightly modified automatic phosphate analyzer (Baykov and Avaeva, 1981) in 3 ml of reaction mixture at 30°C for 5 min. In all experiments substrate cleavage did not exceed 5%. At very low substrate concentrations a coupled assay mixture was used containing 100–500 μg/ml of pyruvate kinase and 200–500 μM phosphoenolpyruvate. Preparations of the ^{18}O-containing reagents, oxygen exchange measurements, and calculations have been described previously by Hackney *et al.* (1979).

Oxygen exchange experiments that used pyruvate kinase for ATP regeneration were performed in a 2.2-ml assay mixture. Initial velocity measurements were made to find the amount of F_1 ATPase necessary for the hydrolysis of about 100 nmol ATP in 20 min. The same assay mixture without F_1 ATPase was used as a control. After incubation for 20 min at 30°C the reactions were stopped by vigorous mixing with an equal volume of chloroform. The control samples were then divided into two 1-ml aliquots and 50 nmol of standard $[^{18}O]P_i$ or $[^{16}O]P_i$ were added to allow measurement of the $[^{16}O]P_i$ or $[^{18}O]P_i$, respectively, that was present initially or during incubation due to the nonenzymic hydrolysis of the reagents. Samples containing F_1 ATPase were treated similarly. One aliquot of each sample then was used directly for the purification of the P_i. $[^{18}O]P_i$ (50 nmol) was added to the other 1-ml aliquot to allow measurement of P_i formed by enzymic hydrolysis of ATP. $[^{16}O]P_i$ or $[^{18}O]P_i$ present in appropriate controls was subtracted from the distribution pattern of the samples containing F_1 ATPase.

Results

Activation of the F_1 ATPase by Bicarbonate Ions

Prior to measurement of the effects on oxygen exchange, the influence of bicarbonate ions on the rate of ATP hydrolysis was measured. At ATP concentrations of 1–10 μM, 0.5–1.5 mM bicarbonate gave a half-maximal effect (data not shown). A bicarbonate concentration of 10 mM was used for most experiments. Data on the initial velocity for the ATP concentrations from 10 μM to 1 mM at 2 or 10 mM bicarbonate concentrations are presented as Eadie–Hofstee plots in Fig. 1. The results are similar to those obtained by Ebel and Lardy (1975). They show an apparently complete elimination of the cooperative effects of the ATP by 10 mM bicarbonate.

Measurements of water oxygen incorporation into P_i formed from $[\gamma\text{-}^{18}O]$ATP in the presence or absence of bicarbonate were first performed at 5 and 10 μM ATP concentrations. According to the data of Choate *et al.* (1979) in this concentration range the intermediate oxygen exchange is relatively high and would be expected to be sensitive to possible changes in O/P and P_c parameters. The results of the oxygen exchange experiments are presented in Table I. They show that bicarbonate did not change the O/P and P_c values. Measurements of the $[^{18}O]P_i$ species formed (data not shown) gave a homogeneous distribution as expected for P_i formation by a single reaction pathway. Clearly the bicarbonate activation does not change the relative values of rate constants governing the phosphate oxygen exchange.

The behavior of the F_1 ATPase at less than micromolar ATP concentrations, as studied by Gresser *et al.* (1982) and Grubmeyer *et al.* (1982), would

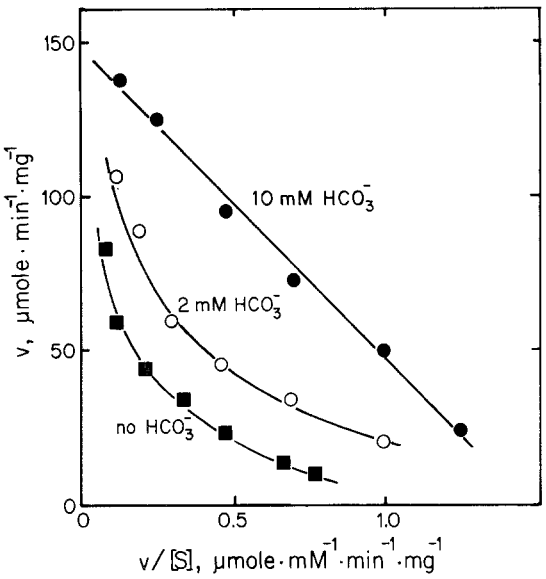

Fig. 1. An Eadie–Hofstee plot for velocity from 10 μM to 1 mM ATP with and without HCO_3^- present. The rates of ATP hydrolysis were measured at 30°C in a 3.0-ml reaction mixture containing 30 mM Tris-acetate, pH 7.6, 30 mM K-acetate, 5 mM Mg-acetate, 0.2 mM NADH, 3 mM phosphoenolpyruvate, 25 μg/ml lactate dehydrogenase, and 50 μg/ml pyruvate kinase. The reaction was monitored at 340 nm for 10 min after the addition of F_1 ATPase.

Table I. The Influence of HCO_3^- on the Velocity of ATP Hydrolysis and on Water Oxygen incorporation into P_i Formed[a]

ATP concentration	nmol P_i formed		O/P[b] ratio		P_c[c]	
	No HCO_3^-	10 mM HCO_3^-	No HCO_3^-	10 mM HCO_3^-	No HCO_3^-	10 mM HCO_3^-
μM						
10	104	256	1.49	1.54	0.44	0.47
5	72	118	1.84	1.82	0.61	0.60

[a] A 2.2-ml reaction volume at 30°C and pH 7.6 contained initially 5 or 10 μM ADP, 300 μM [^{18}O]phosphoenol pyruvate (86% ^{18}O in the phosphoryl group), 30 mM Tris-acetate, 30 mM K-acetate, 5 mM Mg-acetate, and HCO_3^- as indicated. After a 10-min incubation to ensure complete conversion of the ADP to [γ-^{18}O]ATP, hydrolysis was initiated by addition of 10 μl containing 400 ng of F_1 ATPase. Samples were then incubated for 20 min to give sufficient P_i for ^{18}O analyses.

[b] Average number of water oxygens in each P_i released.

[c] Partition coefficient, P_c, is the probability that a bound P_i will undergo exchange of an oxygen instead of being released to the medium (Hackney *et al.*, 1979).

Table II. Effect of 10 mM HCO_3^- on the Velocity of F_1 ATPase at Low ATP Concentrations[a]

ATP concentration (μM)	Reaction velocity (μmol/min/mg)		Increase with HCO_3^- (%)
	No HCO_3^-	10 mM HCO_3^-	
100	63	115	84
10	11	19	71
1	1.3	1.8	44
0.5	0.43	0.51	22
0.1	0.15	0.16	6

[a]The steady-state rate of P_i release was measured using a 5 ml volume at pH 7.6 and 30°C containing 30 mM Tris-acetate, 30 mM K-acetate, 5 mM Mg-acetate, 300 μM phosphoenolpyruvate, 250–1250 μg of pyruvate kinase, and from 9 to 460 ng ATPase at high to low ATP concentrations, respectively. Assays were performed as indicated in Experimental.

not be evident in the ATP concentration range used by Ebel and Lardy (1975) or in the experiments shown in Fig. 1. With required sequential site participation, net ATP cleavage at less than micromolar ATP may reflect the release of ADP and P_i from an enzyme form with products tightly bound. At higher ATP concentrations, with considerably faster velocity, release of a loosely bound ADP may become rate limiting. This step could be accelerated by bicarbonate. It was thus of interest to find if bicarbonate accelerated the ATPase reaction when less than micromolar medium ATP was present. Results presented in Table II show clearly that at these very low ATP concentrations the stimulating effect of bicarbonate on the rate of ATP hydrolysis disappears. This means that the biphasic rate behavior seen without bicarbonate (Fig. 1) also occurs with bicarbonate present but at a lower medium ATP concentration. These and other implications are considered in the Discussion.

ITP Modulation of Oxygen Exchange Accompanying ITP Hydrolysis

The modulation by ITP of the oxygen exchange reactions catalyzed by F_1 ATPase was studied over a wide range of ITP concentrations (0.1 μM to 1 mM). Data for the dependence of the O/P ratio and extent of reversal of cleavage of bound ITP on the ITP concentration are presented in Figs. 2a and b. The upper limits obtained for O/P (3.67) and R (29.6) at 0.1 μM ITP are lower than the values obtained with ATP (O'Neal and Boyer, 1984).

Of definite interest is that considerable intermediate oxygen exchange is observed at the high ITP concentration, in contrast to the behavior with ATP. Similar behavior at high ITP concentrations is also observed for CF_1 ATPase isolated from spinach chloroplasts (Kohlbrenner and Boyer, 1982). The results do show that, like ATP, the concentration of medium ITP modulates the exchange reaction accompanying ITP hydrolysis. Such behavior supports the cooperative alternating site mechanism for F_1 ATPase.

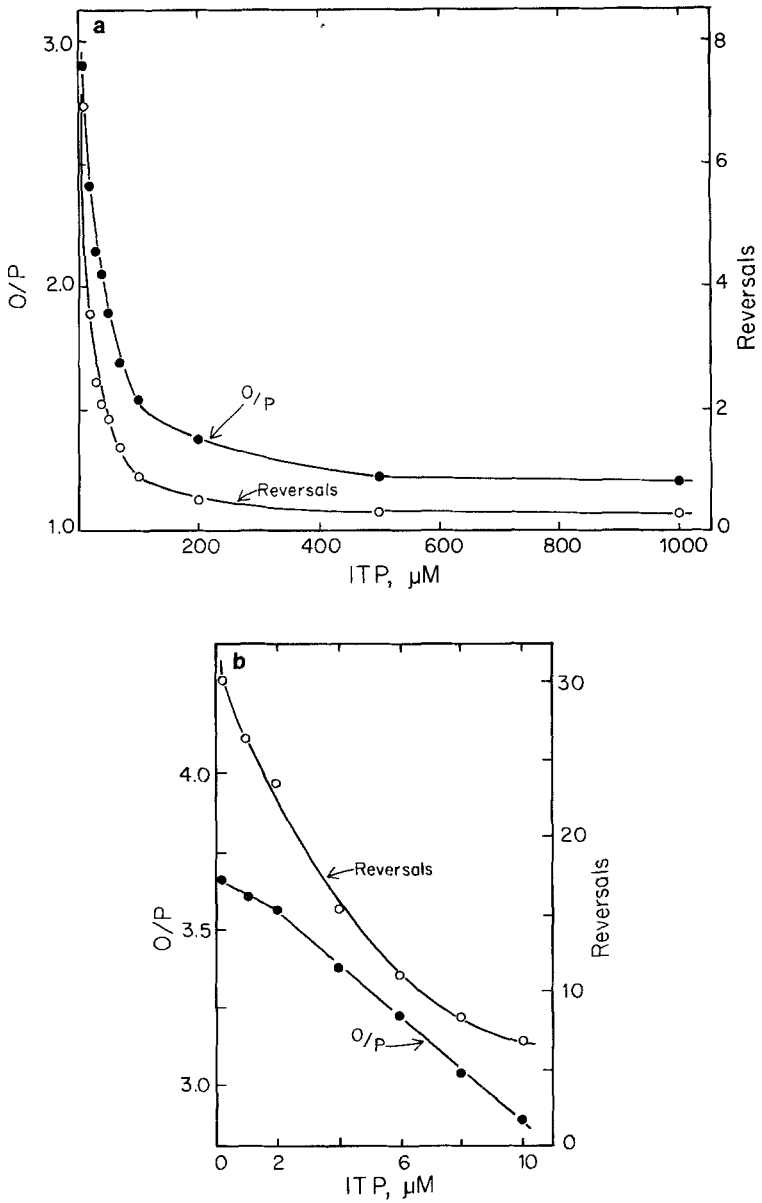

Fig. 2. Effect of ITP concentration on the intermediate phosphate oxygen exchange catalyzed by F_1 ATPase. Conditions were as in Table I, except IDP was used with 300 μM (low IDP) to 2000 μM (high IDP) ^{18}O-phosphoenolpyruvate, 250 μg (high IDP) to 1250 μg (low IDP) of pyruvate kinase, and 200 μg (low IDP) to 0.01 μg (high IDP) of F_1 ATPase. (a) ITP concentrations from 10 μM to 1 mM; (b) ITP concentrations from 0.1 μM to 10 μM.

ITP Modulation of the Intermediate Exchange During ATP Hydrolysis

We readily confirmed the report of Schuster *et al.* (1976) on the activation of ATP hydrolysis by ITP. We obtained results at constant 5 μM or 10 μM ATP concentrations that were similar to theirs. Data for 5 μM ATP are given in Fig. 3. To measure the ITP influence on the intermediate exchange reactions during ATP hydrolysis we used a reaction mixture of relatively large volume (20–40 ml) and [^{32}P]ATP highly enriched with ^{18}O in the γ-phosporyl group. The amount of the ATP hydrolyzed was measured as $^{32}P_i$ formed. The amounts of P_i containing 0–3 atoms of ^{18}O were determined by mass spectrometry, and after correction for unlabeled P_i formed from ITP, the distribution of [^{18}O]P_i species formed from the [γ-^{18}O]ATP was calculated by the method of Hackney *et al.* (1979).

The dependence of the O/P ratio and the extent of reaction reversals on the ITP concentration is presented in Fig. 4. The data show that the extensive reversible cleavage of bound ATP, which otherwise occurs when 5 μM ATP is being hydrolyzed, is sharply reduced when the ITP concentration is increased and ITP hydrolysis also occurs. Further, the increase in ITP is able to nearly completely inhibit the incorporation of more than one water oxygen into P_i formed from ATP at higher ITP concentrations (Fig. 4).

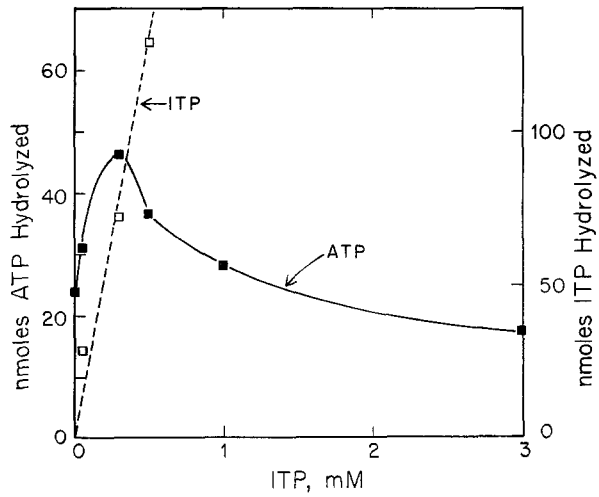

Fig. 3. Effect of ITP on the rate of ATP hydrolysis catalyzed by beef heart F_1 ATPase. The reaction was carried out in a 5-ml reaction mixture for 5 min at 30°C containing 30 mM Tris-acetate buffer, pH 7.6, 5 mM Mg-acetate, 5 μM [γ-^{32}P]ATP, and nonlabeled ITP. The total P_i liberated was measured using the phosphate analyzer. Hydrolysis of [γ-^{32}P]ATP was estimated by P_i separation on a BioRad AG-1-X4 anion exchange column (0.7 × 4 cm) and measurement of the radioactivity.

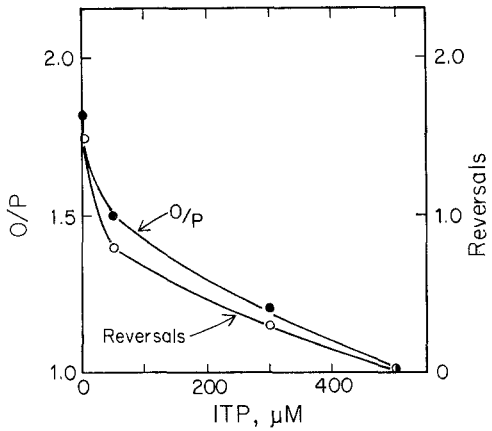

Fig. 4. Effect of ITP concentration on the intermediate ^{18}O exchange during ATP hydrolysis. The reaction was carried out as indicated in Fig. 3 in a 40 ml volume in the presence of 5 μM [γ-^{32}P, ^{18}O]ATP and nonlabeled ITP. [^{16}O]P_i liberated from ITP was subtracted for estimation of the distribution of [^{18}O]P_i species formed from the [γ-^{18}O]ATP.

For the experiment reported in Fig. 4 the distributions of [^{18}O]P_i species formed from the [γ-^{18}O]ATP were as expected if only one catalytic pathway was operative at the various ITP concentrations (data not shown).

Discussion

The striking lack of effect of bicarbonate on the extent of oxygen exchange accompanying ATP hydrolysis establishes that the rate acceleration produced by bicarbonate does not result from an increase in the rate of removal of bound P_i from the oxygen exchange step. This exchange is regarded as resulting from the reversal of hydrolysis of bound ATP (Gresser *et al.*, 1982; Kohlbrenner and Boyer, 1983; Boyer *et al.*, 1982). At less than micromolar concentrations of ATP, bound ADP and P_i formed from ATP accumulate at a catalytic site as shown by the continued oxygen exchange (Choate *et al.* 1979), measurements of the pyruvate kinase-inaccessible ADP (Gresser *et al.* 1982), and the determination of bound products with excess enzyme present (Grubmeyer *et al.*, 1982). Catalysis appears to be occurring at a single site, and the release of products is exceptionally slow (Grubmeyer *et al.*, 1979). The lack of bicarbonate acceleration of the net rate of ATP hydrolysis at less than micromolar ATP concentration (Table II) indicates

that bicarbonate does not accelerate ADP departure from the tight site where hydrolysis occurs.

As ATP concentration is increased above micromolar, ATP binds at an additional catalytic site. A rapid binding change occurs that stops oxygen exchange of the bound P_i and favors product dissociation. At higher ATP concentration during steady-state ATP hydrolysis, all three catalytic sites are filled. When bound P_i is formed the subsequent binding change is rapid, and only slightly greater than one water oxygen is incorporated into each P_i released. Now, as suggested by the model of Gresser *et al.* (1982), dissociation of the more loosely bound P_i or ADP contributes considerably to rate limitation. Bicarbonate acceleration of such dissociation would not change the extent of oxygen exchange for each ATP molecule cleaved. This is in accord with our experimental findings.

Present data do not establish whether the release of P_i or of ADP occurs more rapidly when hydrolysis is occurring at high ATP concentrations and after the binding change has occurred. The considerably greater ability of ADP than of P_i to inhibit net hydrolysis is consistent with ADP having greater affinity and departing last. An attractive explanation for the bicarbonate acceleration is that it may bind to the catalytic site after P_i has departed and accelerate the departure of bound ADP.

That bicarbonate may promote the reaction rate by aiding ADP dissociation was recognized by Ebel and Lardy (1975), and is in harmony with the suggestion of Harris *et al.* (1981) that dissociation of ADP may contribute to rate limitation in the absence of bicarbonate. A very rapid binding change at high medium ATP concentration, followed by a slower bicarbonate-accelerated ADP dissociation, gives a satisfactory correlation of the alternating site mechanism with other findings.

The results with hydrolysis of ITP can also be satisfactorily correlated with, and, indeed, give additional support to the alternating site-mechanism of F_1 ATPase. The data of Fig. 2a show that during hydrolysis of ITP by F_1 ATPase the extent of water oxygen incorporation into the P_i released increases as the ITP concentration decreases. This is as anticipated for cooperative sequential site participation as observed during ATP hydrolysis by F_1 ATPase (Gresser *et al.*, 1982) and for ITP hydrolysis by CF_1 ATPase (Kohlbrenner and Boyer, 1983). From this result it seemed plausible that during the hydrolysis of low concentrations of [γ-^{18}O]ATP the medium ITP concentration would inhibit the ^{18}O exchange that otherwise occurs. The data of Fig. 4 show this to be so. The sequential site mechanism also explains how added ITP can, under some conditions, accelerate the hydrolysis of ATP. Two potential catalytic sites may be filled by increasing the substrate concentration above 1 μM ATP. ATP could bind more readily than ITP to one of the two vacant catalytic sites. If there is insufficient medium ATP to bind to the second

catalytic site, with a higher K_d, the net hydrolysis rate is slow. But with sufficient ITP present the ITP can bind to the second site and considerably accelerate the release of ADP and P_i formed from ATP from the first site, resulting in an empty catalytic site. Either ATP could have a higher affinity for this empty site when ITP is bound at another site, or the reversible association of ITP could allow its replacement by the more favorably bound ATP. Such events would result in the observed increase in cleavage of ATP. When ITP concentration is increased further, however, preferential combination of ATP at all stages is blocked and only ITP is hydrolyzed.

Schuster *et al.* (1976) recognized that the ITP effects they observed could be accounted for by cooperative effects of multiple catalytic sites. Our findings add to this possibility and extend their interpretation. Our results are also in harmony with the findings of Sorgato *et al.* (1982) that ITP, like ATP, can generate a membrane potential in submitochondrial particles. Both ATP and ITP can drive the binding change regarded as conformationally linked to H^+ transport (Gresser *et al.*, 1982; Kohlbrenner and Boyer, 1982; Boyer *et al.*, 1982).

It is of interest that during ITP hydrolysis at high ITP concentrations more residual oxygen exchange remains than observed during ATP hydrolysis at high ATP concentrations. Some additional understanding of this behavior is provided by the observation that high ITP concentrations can stop nearly all the ^{18}O exchange accompanying concomitant hydrolysis of nonlabeled ITP

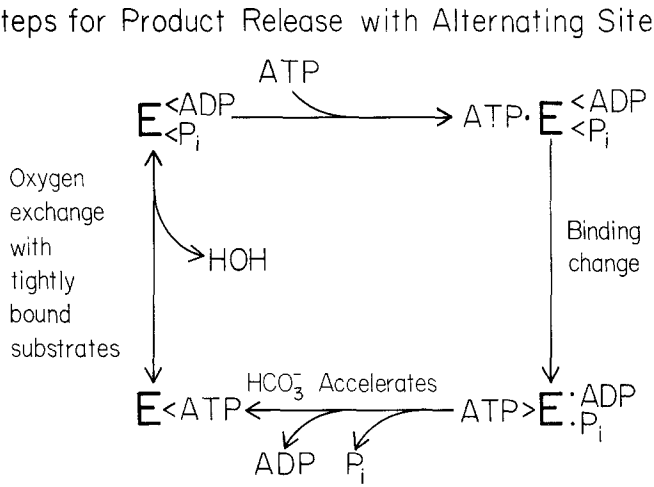

Fig. 5. A depiction of bicarbonate effects with sequential participation of catalytic sites. The center dot indicates a loosely bound substrate and the > a tightly bound substrate. Only two of three sites that likely participate in sequence are depicted in this scheme.

and [γ-^{18}O]ATP (Fig. 4). This suggests that the binding change which terminates the oxygen exchange occurs more readily when bound ADP + P_i rather than when bound IDP + P_i are present at another catalytic site. A diagram indicating these relationships is given in Fig. 5. The modulation of oxygen exchanges by ITP concentration provides further evidence that such modulation reflects binding at catalytic rather than noncatalytic sites. Evidence has been presented that the noncatalytic sites show preferential binding of adenine nucleotides (Harris, 1978; Recktenwald and Hess, 1980; Senda *et al.*, 1983; Di Pietro *et al.*, 1983). Thus the control of oxygen exchange by ITP most likely results from binding to catalytic sites and not from binding to noncatalytic sites. This adds to the considerable evidence that catalytic sites and not control sites are responsible for the velocity and oxygen exchange properties of F_1 ATPase that are modulated by ATP concentration.

Acknowledgments

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