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Kinetics of ATP Synthesis in Pea Cotyledon Submitochondrial Particles[1]

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DARA L. MELANSON[2] AND MARY S. SPENCER[3]
Department of Plant Science, University of Alberta, Edmonton, Alberta, Canada, T6G 2H1

ABSTRACT

A kinetic study of oxidative phosphorylation by pea submitochondrial particles gave two K_m values for ADP, one low, the other high. The high value probably reflected a damaged site or a population of leaky mitochondria. Only the high affinity site with a low K_m for ADP was involved in ATP synthesis. α,β-Methylene ADP was found to be a competitive inhibitor of ATP synthesis. The inorganic phosphate analog, thiophosphate, decreased the apparent K_m of ADP while the rate of the reaction remained approximately the same. Adenyl imidodiphosphate, a specific inhibitor of ATP hydrolysis activity, had little effect on oxidative phosphorylation. A slight decrease in the K_m of the high affinity binding site for ADP was noted. Aurovertin was found to be a potent inhibitor of oxidative phosphorylation in pea submitochondrial particles. The K_m of the high affinity site was increased 10-fold. Also, the inhibition normally exerted by ADP on ATPase activity was severely reduced by aurovertin. In contrast, increasing the concentration of aurovertin only slightly affected the level of inhibition caused by adenyl imidodiphosphate on ATP hydrolysis.

The mitochondrial ATPase-ATP synthetase complex both synthesizes and hydrolyzes ATP. The enzyme consists of three parts: F_1, a water-soluble protein that contains catalytic and allosteric sites; F_0, a water-insoluble, integral membrane protein complex that contains a protein pore and possibly functions as an energy transducer; and the stalk protein(s), currently thought to hold F_1 and F_0 together. It has been suggested that energy-dependent conformational changes that alter the affinities of adenine nucleotide binding sites on F_1 are critical features of oxidative phosphorylation (1, 4). Several attempts have been made to elucidate the chemical natures and numbers of these binding sites. For instance, Penefsky *et al.* (21), proposed that at least five adenine nucleotide binding sites exist for beef heart ATPase and three of these are "tight" or slow reacting. Although direct participation of these slower sites in ATPase enzyme activity may be excluded, they could affect activity by allosteric interactions (8). With reference to the remaining two sites, K_m values as low as 0.28 μM have been reported for the binding of ADP (9).

There is the interesting possibility that the F_1 portion of the ATPase enzyme contains at least two catalytic sites, one for ATP synthesis and one for ATP hydrolysis. The antibiotic aurovertin is generally recognized as a potent inhibitor of oxidative phosphorylation but has little effect on the reverse, hydrolytic reaction (12). Also, experiments with aurovertin have shown that the binding of ATP alters the conformation of the enzyme, which could preclude the approach of ADP to the catalytic site (11). Although such results indicate that aurovertin may act by binding at or near the ADP site, leaving the affinity of the enzyme for ATP unaffected, they do not necessarily mean there are two separate sites for ADP and ATP. Also, it has been suggested that the release of newly synthesized ATP requires an energy-dependent conformation change at the catalytic site (1). This too could occur by a one- or two-site mechanism.

A study of the kinetics of oxidative phosphorylation may help to resolve the two-site question, as it may be used to determine the order of binding and release of substrates and products. The present study comprises a kinetic analysis of the synthesis of ATP by pea SMP[4] in the presence of various inhibitors of oxidative phosphorylation and ATP hydrolysis. Substrate analogs that resemble the natural substrate sufficiently well to interact with the active site on the enzyme are used to provide a new insight into the mechanism of ATP synthesis. Two inhibitors of oxidative phosphorylation, the Pi analog, thiophosphate, and the ADP analog, α,β-methylene ADP are employed to investigate the nature of the binding site for ADP. The ATP analog, AMP-PNP, a potent inhibitor of ATP hydrolysis activity, is used to assess the possibility of two separate sites for ADP and ATP. The use of SMP, for the studies reported here, is advantageous in that substrates in solution have direct access to the catalytic site on the enzyme complex.

MATERIALS AND METHODS

Pea seeds (*Pisum sativum* L. cv. Homesteader) were germinated at 27 C in the dark for 4 days in vermiculite. Mitochondria were prepared by density gradient centrifugation from approximately 1,200 ml of harvested cotyledons as previously described (25). They were then resuspended in 60 ml of ice cold "SMP buffer" (0.25 M sucrose, 50 mM Tes, brought to pH 7.0 at 20 C with 1 M KOH) and SMP were prepared by sonication as previously described (7). Removal of a small amount of unbroken mitochondria from the sonicate was achieved by low speed centrifugation and the translucent supernatant layer was further centrifuged at 100,000*g* to sediment the SMP (7). The pellet (SMP) was resuspended in SMP buffer to a concentration of 10 to 15 mg protein/ml. The SMP were stored in 0.3-ml aliquots in stoppered glass tubes at −20 C for no longer than 2 weeks, since the oxidative phosphorylation capability of the SMP declined rapidly thereafter.

ATP synthesis was measured by the esterification of [^{32}P]Pi. The reaction was in a total volume of 1 ml. The basic assay medium was: 0.3 M sucrose, 4 mM $MgSO_4$, 20 mM glucose, 50 mM Tes, brought to pH 7.2 with 1 M KOH at 25 C. Just prior to the

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[2] Present address: Department of Biology, University of Calgary, Calgary, Alberta T2N 1N4.

[3] To whom reprint requests should be addressed.

[4] Abbreviations: SMP, submitochondrial particles; AMP-PNP, adenyl imidodiphosphate.

assay, the medium was adjusted to contain 5 units of hexokinase, ADP concentrations ranging from 0.02 to 1.0 mM, 1.5 mM NADH and 2.5 mM neutralized K_2HPO_4 (containing approximately 200,000 cpm [^{32}P]Pi). In addition, various inhibitors of oxidative phosphorylation and ATP hydrolysis were added to the appropriate tubes. Except where noted, blank tubes contained all assay components plus 1 μg oligomycin. The reaction was started either by the addition of 75 μg SMP protein to the complete medium or by the addition of ADP to medium already containing the SMP. The tubes were incubated for 6 min at 25 C and ATP synthesis[5] was measured as previously described (7).

Hydrolysis[5] of ATP was monitored by the release of phosphate (6, 16). The assay medium consisted of 0.3 M sucrose, 4 mM $MgSO_4$, 50 mM Tes, 20 mM glucose, and 3 mM ATP, brought to pH 7.2 at 20 C with 1 M KOH. The reaction was run for 10 min at 25 C.

Protein was measured by a dye-binding procedure (24) which gave results similar to the method of Lowry *et al.* (15).

The experiments described were run several times as it was not possible, because of the number of samples involved, to have replicates within each experiment. The data presented are, therefore, from representative runs. Variation in the activity between experiments reflected both different preparations of SMP and different storage times.

All biochemicals were obtained from Sigma. Baker's yeast hexokinase was Sigma product H-4502 (200–300 units/mg protein). Aquasol and [^{32}P]Pi in 20 mM HCl were obtained from New England Nuclear Corp. Thiophosphate was purchased from Alfa Inorganics and all other inorganic chemicals were Fisher certified grade. A 50% pure preparation of aurovertin was generously donated by Dr. H. Penefsky of The Public Health Research Institute of the City of New York, Inc., New York 10016.

RESULTS

Kinetics of ATP Synthesis in Pea SMP. As previously reported by our laboratory (7), a biphasic Lineweaver-Burk curve was obtained for the ADP kinetics of oxidative phosphorylation, rather than a straight line typical of classic Michaelis-Menten kinetics. This suggested that the curve for ATP synthesis was the result of the combined operation of two separate components. Derivation of the apparent Michaelis-Menten kinetic constants by extrapolation of the two straight line segments of the curve to intersect at the *X* and *Y*-axis gave values for K_{m_1} and V_{max_1} (Site 1) of 9 μM and 0.26 units, respectively, and that of K_{m_2} and V_{max_2} (Site 2) to be 30 μM and 0.33 units. (Site 1 will be referred to as a "high affinity site" for ADP and Site 2 as a "low affinity site" for ADP.) When dealing with such curvilinear plots, it is important to realize that the values obtained for Site 1 would contribute to Site 2 and vice versa. Each component of the curve should be examined by itself before extracting kinetic parameters or attempting an inhibitor analysis (5).

It is possible to calculate approximate K_m and V_{max} values from a biphasic Lineweaver-Burk curve given a sufficient number of points (5). To extract the values for the parameters of the separate components from the data for $1/v_1 + v_2$ *versus* 1/[S] it is assumed that the values at very low concentrations arise mainly from the low K_m, Site 1; and those at very high concentrations arise from the high K_m, Site 2. Preliminary corrections may then be applied to Site 2 for the contribution to the rate expected for Site 1 and vice versa. By repeating the procedure several times, stable values for K_m and V_{max} of each site may be found. It is simpler, in view of the number of calculations involved, to computerize the analysis of the separate kinetic parameters (5, 18). (This method has been used successfully in the kinetic study of multiple transport components for dicarboxylic amino acids in *Streptococcus faecalis* [22]). The separate parameters for ATP synthesis in pea SMP (Fig. 2 in ref. 7) were so analyzed by the use of the program of Neal (18). The theoretical curve obtained was used as a basis for adjusting the assumed values for K_{m_1}, V_{max_1}, K_{m_2} and V_{max_2}. The new assumed values of K_{m_1} = 4.8 μM and V_{max_1} = 0.21 units and K_{m_2} = 160 μM and V_{max_2} = 0.12 units reflect separation of the original curve into two separate components, each unaffected by the other. A comparison of the values for V_{max_1} and V_{max_2} suggests that the original curve was the result of about a two-thirds contribution from Site 1 and one-third contribution from Site 2.

Effect of α,β-Methylene ADP on the Synthesis of ATP. The ADP analog, α,β-methylene ADP did not appear, at first, to have any effect on the kinetics of ATP synthesis even when the high concentration of 1.0 mM was allowed to compete with 0.02 mM ADP for the active site (Table I). A computer analysis of the data did, however, resolve the plots and showed that the analog had an inhibitory effect (Table I). α,β-Methylene ADP increased the K_m of Site 1 without altering V_{max_1}. That is, it is a competitive inhibitor of ATP synthesis with respect to ADP. Similar results were obtained by Schuster *et al.* (23) for beef heart SMP. Since the inhibitor had little effect on the K_m of Site 2 in pea SMP this suggests that Site 1 is the normal route for oxidative phosphorylation.

Effect of Thiophosphate on the ADP Kinetics of ATP Synthesis. The Pi analog, thiophosphate, was allowed to compete with 2.5 mM Pi for the normal binding site on the ATPase enzyme (Table II). For the preparation used in these experiments, computer analysis of the data indicated that the ADP kinetic parameters were not separated widely enough to use two Michaelis-Menten

Table I. *Kinetic Parameters of the Effect of 0.5 mM and 1.0 mM α,β-Methylene ADP on ATP Synthesis*

Assay as described under "Materials and Methods." SMP were preincubated for 6 min at 25 C in assay medium with or without 0.5 or 1.0 mM α,β-methylene ADP. The reaction was started by the addition of ADP. Substrate was 1.5 mM NADH. Units are expressed on a per mg protein basis.

Treatment α,β-Methylene ADP	Apparent ADP Kinetics				Calculated ADP Kinetics			
	K_{m_1}	V_{max_1}	K_{m_2}	V_{max_2}	K_{m_1}	V_{max_1}	K_{m_2}	V_{max_2}
mM	*μM*	*units*	*μM*	*units*	*μM*	*units*	*μM*	*units*
Control	3.7	0.36	40.0	0.44	0.85	0.32	255	0.13
0.5	3.7	0.37	30.2	0.43	3.3	0.32	114	0.09
1.0	3.7	0.37	40.4	0.45	2.8	0.33	287	0.13

Table II. *Kinetic Parameters of the Effects of 0.25 mM, 0.5 mM, and 1.0 mM Thiophosphate on ATP Synthesis*

Assay as described under "Materials and Methods" except that the complete medium contained 0, 0.25, 0.5 or 1.0 mM thiophosphate. The reaction was started by the addition of SMP. The substrate was 1.5 mM NADH. Units are expressed on a per mg protein basis.

Treatment Thiophosphate	Apparent ADP Kinetics				Calculated ADP Kinetics	
	K_{m_1}	V_{max_1}	K_{m_2}	V_{max_2}	K_m	V_{max}
mM	*μM*	*units*	*μM*	*units*	*μM*	*units*
Control	2.5	0.13	15.5	0.14	4.5	0.13
0.25	3.8	0.13	13.5	0.14	3.9	0.13
0.5	1.1	0.11	11.6	0.12	2.4	0.11
1.0	1.1	0.11	16.4	0.12	1.5	0.11

[5] One unit of ATP synthetase is defined as the activity required to esterify 1 μmol of Pi/min under the given assay conditions. One unit of ATPase hydrolase is defined as the activity required to hydrolyze 1 μmol ATP/min under the given assay conditions.

equations, so a single equation was used to find the kinetic constants for the entire curve. Also, the apparent K_m of ADP obtained from one Michaelis-Menten equation by computer was similar to that obtained for Site 1 by drawing a tangent to the curve. It was, therefore, assumed that Site 2, because of decreased amounts and/or inactivity, contributed very little to the kinetics of ATP synthesis. The data presented in Table II refer mainly to the high affinity site for ADP. The calculated ADP kinetics show that increasing concentrations of thiophosphate decreased the K_m of the enzyme for ADP.

Effects of the ATP Analog, AMP-PNP, on the Kinetics of ATP Synthesis and Hydrolysis. As found in the thiophosphate experiments, Site 1 was the major contributing factor to the ADP kinetics curve, so the Michaelis-Menten constants refer to the high affinity site (Table III). AMP-PNP, a potent inhibitor of ATP hydrolysis, had a relatively small effect on the ADP K_m of oxidative phosphorylation, an observation made previously for beef heart ATPase (19).

The extent of inhibition of ATP hydrolysis by AMP-PNP was also examined (Fig. 1). At a low concentration (0.05 mM) and in the presence of 3 mM ATP, AMP-PNP inhibited ATPase activity by 30 to 40%. A higher concentration (0.6 mM) inhibited the activity by 50%.

Effects of Aurovertin on ATP Synthesis and Hydrolysis. The antibiotic aurovertin was found to inhibit oxidative phosphorylation in pea SMP (Table IV), similar to its effects on other systems (12, 14). At a concentration of 40 μM, aurovertin increased the K_m of ADP for the high affinity site by about 10-fold.

The results in Figure 2 show that when only 3 mM ATP (control) was added to SMP preincubated with high concentrations of aurovertin (80 and 120 μM) ATPase activity was just reduced by 20%. When, however, the ATP analog AMP-PNP was also included in the preincubation medium, hydrolysis activity was similar to that obtained in the absence of aurovertin (Fig. 1.) The slight reduction in the effectiveness of 0.6 mM AMP-PNP while in the presence of aurovertin may just be a result of preincubation with aurovertin, and so was not considered significant. In contrast, the effect of 5 mM ADP, which is able to inhibit the hydrolysis reaction by 60 to 70% (line 2, Fig. 3) was severly reduced by preincubation with aurovertin (Fig. 2). The 50% decrease brought the value of the test sample close to the level of inhibition obtained by the control. Aurovertin apparently nullified the effect of ADP.

Table III. *Kinetic Parameters of ATP Synthesis in the Presence of 0.2 mM and 0.5 mM AMP-PNP*

Assay as described under "Materials and Methods." SMP were preincubated in assay medium with or without 0.2 or 0.5 mM AMP-PNP for 6 min at 25 C. The reaction was started by the addition of ADP. Substrate was 1.5 mM NADH. Units are expressed on a per mg protein basis.

Treatment AMP-PNP	Apparent ADP Kinetics				Calculated ADP Kinetics	
	K_{m_1}	V_{max_1}	K_{m_2}	V_{max_2}	K_m	V_{max}
mM	*μM*	*units*	*μM*	*units*	*μM*	*units*
Control	4.0	0.20	21.8	0.22	5.0	0.21
0.2	1.8	0.18	16.7	0.21	4.7	0.20
0.5	1.9	0.19	19.2	0.21	4.0	0.20

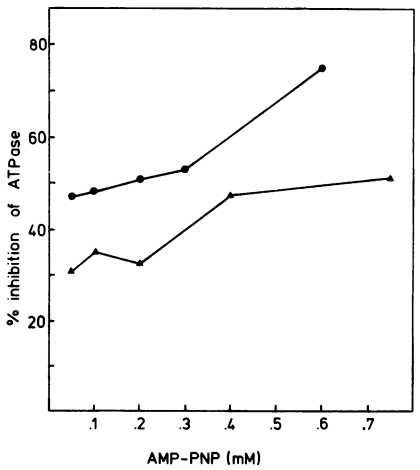

FIG. 1. Inhibition of ATP hydrolysis by AMP-PNP. Assay as described under "Materials and Methods". SMP were preincubated at 25 C for 6 min in the presence or absence of 0.05, 0.1, 0.2, 0.3, 0.4, 0.6 or 0.75 mM AMP-PNP. The reaction was started by the addition of 3 mM ATP and allowed to proceed for 10 min. Sample tubes not containing SMP and those containing SMP and oligomycin were used to correct for nonenzymic hydrolysis of ATP and AMP-PNP (and oligomycin-insensitive enzymic ATPase). ●, experiment 1; ▲, experiment 2.

DISCUSSION

Similar K_m (ADP) values to those calculated by computer for pea SMP have been found for purified inner membrane vesicles

Table IV. *Kinetic Parameters of the Effect of Aurovertin on the Synthesis of ATP*

Assay as described under "Materials and Methods." SMP were preincubated for 6 min at 25 C in assay medium with or without 20 or 40 μM aurovertin. The reaction was started by the addition of ADP. Units are expressed on a per mg protein basis.

Treatment Aurovertin	Apparent ADP Kinetics				Calculated ADP Kinetics			
	K_{m_1}	V_{max_1}	K_{m_2}	V_{max_2}	K_{m_1}	V_{max_1}	K_{m_2}	V_{max_2}
μM	*μM*	*units*	*μM*	*units*	*μM*	*units*	*μM*	*units*
Control	7.1	0.36	29.8	0.43	0.53	0.26	98.0	0.17
20	6.8	0.34	23.3	0.39	1.8	0.28	124.0	0.12
40	6.9	0.34	31.8	0.40	5.4	0.32	400.0	0.10

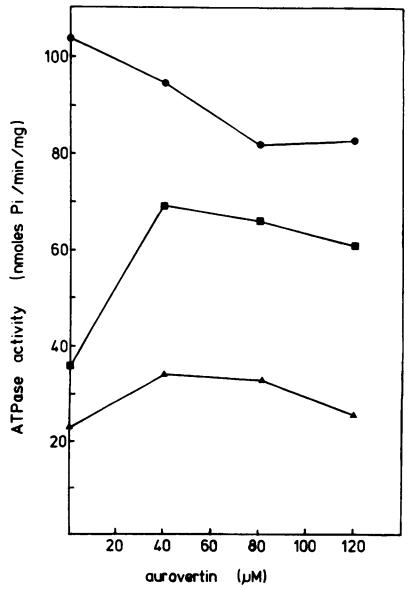

FIG. 2. Effect of ADP and AMP-PNP on ATP hydrolysis of aurovertin treated SMP. Assay as described under "Materials and Methods". SMP were preincubated with aurovertin for 6 min at 25 C in ATPase medium with or without 5 mM ADP or 0.5 mM AMP-PNP. The reaction was started by the addition of 3 mM ATP and allowed to proceed for 10 min. ●, control; ▲, ADP; ■, AMP-PNP.

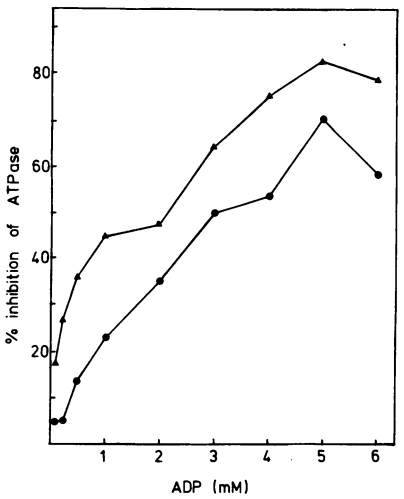

FIG. 3. Inhibition of ATP hydrolysis by ADP. Assay as described under "Materials and Methods". Line 1 (▲). The medium contained both ADP and 3 mM ATP and the reaction was started by addition of SMP. Line 2 (●). SMP were preincubated at 25 C for 6 min in the presence of ADP. The reaction was started by the addition of 3 mM ATP. In both cases the reaction was terminated after 10 min. Blank tubes not containing SMP were used to correct for non-enzymic hydrolysis of ATP and ADP.

of rat liver (3) and for beef heart SMP (23) but the results were linear. In pea SMP there was a second, higher K_m for ADP, termed Site 2 or the "low affinity site." This K_m varied, by computer analysis, from 98 to 255 μM. In some experiments, the apparent K_m of ADP for this site was not vastly different from that for Site 1, which not only made a kinetic analysis by two Michaelis-Menten equations impossible but also suggested that Site 2, as a separate process from Site 1, contributed very little to the kinetics of ATP synthesis. While it is possible that a second site may exist, the fact that the K_m values and their relative proportions varied from batch to batch and with storage suggested that Site 2 is a damaged site or reflects a population of leaky SMP. Such a site or population would likely have a higher K_m for ADP than normal. This might explain why Bygrave and Lehninger (2) obtained a single high K_m for ADP of 300 μM for sonicated rat liver mitochondria. Kayalar *et al.* (10) have shown that uncoupling by 2,4-dinitrophenol increased the apparent K_m of beef heart SMP for ADP and Pi. In addition, since the K_m of the "low affinity Site 2" in pea SMP was so variable, it did not appear likely that it represented one of the multiple binding sites proposed by Penefsky *et al.* (21). Previous work (7) showed that the biphasic curve did not result from the presence of undisrupted mitochondria or noneverted particles. We have concluded that ATP synthesis, under normal physiological conditions, has a low K_m for ADP and is represented here as Site 1.

Although α,β-methylene ADP was shown to be a competitive inhibitor of ATP synthesis with respect to ADP in the pea SMP system, it only increased the K_m of Site 1 3-fold. Since it was necessary to use high concentrations to elicit a response, this compound did not appear to be an effective inhibitory analog of ADP. Larsen *et al.* (13) have reported that α,β-methylene ADP has a significantly different conformational shape from ADP, which could prevent binding of this compound to the active site. However, Schuster *et al.* (23) have shown α,β-methylene ADP to be an effective inhibitor of ATP synthesis in beef heart SMP.

Thiophosphate was shown to decrease the K_m for the ADP kinetics. A more in-depth study by Schuster *et al.* (23) of the effect of thiophosphate on the ADP kinetics in beef heart SMP showed this Pi analog to be a noncompetitive inhibitor of ATP synthesis with respect to ADP. It was concluded from their results that the events in ATP synthesis are ordered, with Pi binding to the enzyme before ADP. In the experiments with pea SMP, if thiophosphate competes with Pi, the amount of Pi seen by the enzyme would effectively be lowered, and the K_m ADP raised. Since K_m ADP is lowered (without effect on V_{max}), thiophosphate may be acting as an allosteric activator for F_1. Penefsky (20) has suggested that Pi has a separate binding site from ADP and has raised the possibility that the Pi binding site is in the position occupied by the γ-phosphate group of ATP. Thiophosphate may bind at a site near to the normal Pi binding site and induce a shift in the conformational and energetic state of the enzyme to that of oxidative phosphorylation.

Alternatively, thiophosphate could bind to the normal Pi-binding site in a rapidly reversible fashion and achieve the same result. The suggestion that phosphate could activate the proton-translocating mechanism of the ATPase system was proposed earlier by Mitchell and Moyle (17) for rat liver and may hold for the phosphorylation of ADP by pea SMP.

The ATP analog, AMP-PNP had very little effect on oxidative phosphorylation in pea SMP (Table III). The K_m for ADP was only slightly decreased indicating that this ATPase inhibitor did not compete with ADP for the active site. AMP-PNP did, however, inhibit ATP hydrolysis activity by 50%. Other researchers have shown AMP-PNP to be an unusual inhibitor that blocks the hydrolysis reaction but exhibits little inhibition of the ATP synthesis reaction of the same enzyme (12, 14). We could interpret our results to indicate that two separate sites exist, one for ATP synthesis and the binding of ADP and one for ATP hydrolysis and the binding of ATP. It is still possible, however, that the results arise from kinetic effects whereby the conformational and/or energetic state of the enzyme during oxidative phosphorylation may preclude the approach of AMP-PNP to the catalytic site that interacts with ADP and Pi.

To investigate the problem in more detail, the effects of aurovertin on ATP synthesis and hydrolysis were studied. This antibiotic inhibited ATP synthesis in the pea SMP system but had little effect on the hydrolysis activity, an observation made by researchers with other systems (12, 14, 17). Aurovertin has also been used as a fluorescent reporter molecule of conformational changes induced by ATP (4, 14) and Mitchell and Moyle (17) have proposed from their results that aurovertin increased the affinity of membrane bound F_1 for ATP and decreased that for ADP. A similar effect of pea SMP was found when the usual levels of inhibition by ADP and AMP-PNP on ATP hydrolysis were compared with those obtained from aurovertin treated SMP. The inhibition of hydrolysis by 0.6 mM AMP-PNP was slightly decreased in the presence of a low level of aurovertin but then increased to its former value with greater concentrations. Conversely, the inhibition normally caused by 5 mM ADP was reduced by 50%. This indicates that aurovertin lowered the affinity of the enzyme for ADP. It also suggests that ADP exerts its inhibition on ATP hydrolysis by binding at a site other than that for ATP. Penefsky (19) has pointed out that the ADP binding constants in the noncatalytic site of ATPase and the Michaelis constants for ADP during oxidative phosphorylation are very close. So, if aurovertin does bind at the ADP site it is probably the mode of action of the antibiotic in preventing oxidative phosphorylation.

Penefsky *et al.* (21) have proposed that the differential effects of aurovertin on ATPase activity and oxidative phosphorylation could be interpreted in terms of an effect of the antibiotic on the affinity of F_1 for ATP and ADP. Furthermore, this could indicate that the energetic state for synthesis or hydrolysis may depend on conformational changes induced by the particular nucleotide. A 2-site mechanism for the action of aurovertin is possible by the binding of aurovertin to the site that normally accepts ADP, leaving the ATP site open. In view of the multiple adenine nucleotide binding sites on coupling ATPases, a more complex route of phosphorylation might occur than just the reversal of the

ATP hydrolytic reaction. It is possible that two catalytic sites exist, one for ATP hydrolysis and sensitive to AMP-PNP and one for ATP synthesis and insensitive to AMP-PNP.

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