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Interactions between the Mitochondrial Adenosinetriphosphatase and Periodate-Oxidized Adenosine 5′-Triphosphate, an Affinity Label for Adenosine 5′-Triphosphate Binding Sites†

Peter N. Lowe* and R. Brian Beechey

ABSTRACT: Periodate-oxidized ATP (o-ATP) was prepared as an affinity label of nucleotide binding sites on the chloroform-released ox heart mitochondrial ATPase. In the presence of $MgSO_4$, o-ATP is a substrate for the ATPase. It can act as a reversible, competitive inhibitor of ATPase activity and can also induce an irreversible inhibition of ATPase activity. In parallel with the irreversible inhibition, covalent incorporation of [^{3}H]o-ATP occurs. ATPase has about 1.05 mol of o-ATP bound per mol of ATPase when the enzyme is 50% inhibited. Most of the covalently bound o-ATP is associated with the α and β subunits and is equally distributed between them. The incorporation of o-ATP into the ATPase is reduced, and the irreversible inhibition induced by o-ATP can be prevented totally by MgADP, MgATP, EDTA/ATP, or EDTA. The location, number, and the functional significance of the o-ATP binding sites are discussed. o-ATP can decompose to form an adenine-containing compound and the tripolyphosphate anion in a β-elimination reaction mechanism. The structures of the adenine-containing compound and its borohydride reduction product were determined. The adenine-containing elimination product inhibited the mitochondrial ATPase activity at a rate greater than that observed with o-ATP. The nature and mechanism of the inhibition of ATPase activity exerted by o-ATP and the elimination product were examined. The significance of the β-elimination reaction to the use of periodate-oxidized nucleotides as affinity labels of nucleotide binding sites on other proteins is discussed.

A common feature of preparations of mitochondrial ATPase (Senior, 1979) is that they possess multiple nucleotide binding sites (Harris, 1978). One or more of these may be catalytic; others may serve regulatory or structural roles (Slater et al., 1979). Although a number of nucleotide analogues have been used to affinity label nucleotide binding sites on the soluble heart mitochondrial ATPase, few, if any, are confirmed catalytically active site directed inhibitors (Russell et al., 1976; Drutsa et al., 1979; Slater et al., 1979; Di Pietro et al., 1979; Lunardi & Vignais, 1979; Kozlov & Milgrom, 1980). This is because either they are not substrates or the labeling has been carried out under conditions where the hydrolysis of ATP does not occur, e.g., in the absence of Mg^{2+}.

The application of periodate-oxidized ATP (o-ATP)[1] to the labeling of sites on the ox heart mitochondrial ATPase has been reported briefly (Lowe et al., 1979b). In this paper, we extend this work. We present results suggesting that o-ATP may label specifically a catalytic site (1 mol/mol of ATPase)

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[1] Abbreviations: o-ATP, o-ADP, o-AMP, o-adenosine, and o-CTP, periodate-oxidized ATP, ADP, AMP, adenosine, and CTP, respectively; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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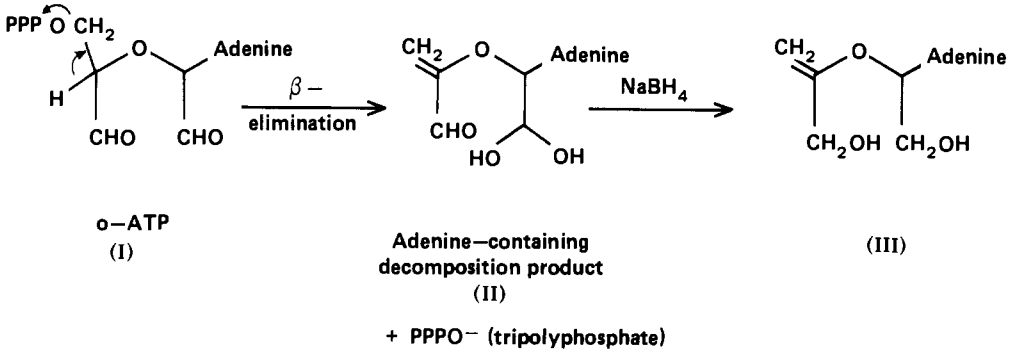
Scheme I



as well as a noninhibitory site (1 mol/mol of ATPase). The latter may represent a uniquely reactive amino group.

Periodate-oxidized nucleotides have been used increasingly frequently as affinity labels of nucleotide binding sites on proteins [see Lowe (1980) for references]. Although these compounds are easy to prepare, they are not very stable under conditions often used to label proteins. We show that o-ATP can decompose to form an adenine-containing compound (compound II, Scheme I) and the tripolyphosphate anion. The general significance of this degradation to the use of periodate-oxidized nucleotides as affinity labels is discussed with particular reference to the mitochondrial ATPase.

Experimental Procedures

Preparation of ATPase and Measurement of ATPase Activity. Chloroform-released mitochondrial ATPase was prepared and its activity assayed (using a coupled enzyme assay) as described by Lowe et al. (1979c). In this paper, the term ATPase refers to this chloroform-released preparation, unless otherwise stated. The protein content of the preparations either was measured directly (Beechey et al., 1975) or was measured after trichloroacetic acid precipitation (Peterson, 1977). Bovine serum albumin was used as a standard. The molecular weight of the ATPase molecule was taken to be 360 000.

Sources of Compounds. Efrapeptin D was a gift from Dr. C. G. Jackson. Aurovertin B was prepared by Dr. P. E. Linnett. [α-^{32}P]ATP, sodium salt (13.9 Ci/mol), was obtained from Amersham. [2,8-$^{3}H_2$]ATP, tetrasodium salt (25–32.5 Ci/mol), was obtained from New England Nuclear.

Polyacrylamide Gel Electrophoresis. This was carried out by using 10% gels containing 0.1% $NaDodSO_4$ as described by Weber et al. (1972), except that the buffer was 0.1 M H_3PO_4/Tris, pH 6.8. The sample (2 mg of protein/mL) to be electrophoresed was mixed with an equal volume of sample buffer. This contained 2% (w/v) $NaDodSO_4$, 2% (v/v) 2-mercaptoethanol, and 0.2 M H_3PO_4/Tris, pH 6.8. To each 100 μL of this mixture was added 25 μL of a solution containing 0.05% (w/v) bromophenol blue and 1% (v/v) mercaptoethanol dissolved in glycerol. The mixture was heated to 100 °C for 3 min before being applied to the gel.

Radioactivity on gels was measured by cutting the gel into slices and eluting with 0.8 mL of 1% (w/v) $NaDodSO_4$. The mixture was incubated overnight before addition of scintillation fluid.

Measurement of Radioactivity. Radioactivity was measured by using a Packard Tri-Carb 3255 scintillation counter. Samples were included in 10 mL of Packard MI-99 scintillation fluid. Tritium and carbon-14 count per minute data were converted into disintegrations per minute by using a channels ratio method calibrated by standards quenched with chloroform. The efficiencies of counting tritium and carbon-14 were 30–45% and 80–90%, respectively.

Assay of Fluorescence. Fluorescence was measured on a Baird-Atomic Model SFR 100 ratio recording spectrofluorometer working in the ratio mode. The excitation wavelength was 368 nm, and the emission wavelength was 470 nm.

Measurement of Ligand Binding to the ATPase by Perchloric Acid Precipitation. ATPase protein was precipitated from a mixture of o-ATP by addition of $HClO_4$ to a final concentration of 4%. The mixture was left on ice for 10 min and then centrifuged on a Hawksley Microhaematocrit centrifuge 10 min. The pellet was washed twice by suspension in 4% (v/v) $HClO_4$ and then dissolved in 100 mM Tris/1% $NaDodSO_4$ (w/v) by warming. The amount of radiolabel associated with this solution was then estimated and the binding stoichiometry to the ATPase estimated, assuming quantitative precipitaiton of the ATPase protein [cf. Peterson (1977)]. In some experiments bovine serum albumin was added to a final concentraiton of 4.4 mg of protein/mL to the solution of the ATPase before the addition of $HClO_4$. The results were identical.

Centrifuge Gel Filtration. ATPase (0.1–0.2 mL) was applied to a column, in a 1-mL plastic syringe, of Sephadex G-50 (fine) which had been equilibrated with 10 mM Hepes/KOH, pH 7.6. The column was then centrifuged as described by Penefsky (1977).

Incorporation of o-ATP into ATPase Pretreated with Other Ligands. (A) Pretreatment with Efrapeptin or Adenylyl 5′-Imidodiphosphate. ATPase (100 μg/mL) was incubated in 50 mM barbituric acid/NaOH, 5 mM $MgSO_4$, and 10% (v/v) methanol, pH 9.1, or in 50 mM $Na_2B_4O_7$, 5 mM $MgSO_4$, and 10% (v/v) methanol, pH 9.0 at 30 °C, for 1 h with either 12 μM efrapeptin D or 167 μM adenylyl 5′-imidodiphosphate. [^{3}H]o-ATP (124 Ci/mol) was then added to a final concentration of 5 μM. The mixtures were incubated at 35 °C, and at intervals, samples were removed for assay of covalent incorporation of radiolabel. In the barbiturate buffer, the inhibitions of ATPase activity induced by efrapeptin D and adenylyl 5′-imidodiphosphate were 95% and 100%, respectively. In the borate buffer, the inhibitions were 55% and about 60%, respectively. Rates of incorporation of radiolabel were the same in either buffer.

(B) Pretreatment with Phenylglyoxal. ATPase (0.5 mg/mL) was incubated with 5 mM phenylglyoxal in 50 mM $Na_2B_4O_7$, 5 mM $MgSO_4$, and 10% (v/v) methanol, pH 8.0. Samples (0.16 mL) were removed when the ATPase activity was inhibited by either 56% or 84% and were applied to centrifuge gel filtration columns. The eluates were diluted with 50 mM $Na_2B_4O_7$, 5 mM $MgSO_4$, and 10% (v/v) methanol, pH 9.1, to a protein concentration of 100 μg/mL. [^{3}H]o-ATP (125 Ci/mol) was added to a final concentration of 5 μM, and the incorporation of radiolabel into the ATPase was followed.

(C) Pretreatment with 7-Chloro-4-nitro-2,1,3-benzoxadiazole. ATPase (6 μM) was treated with 100 μM 7-

chloro-4-nitro-2,1,3-benzoxadiazole until the ATPase activity was inhibited by 96%, as described by Ferguson et al. (1974). Excess reagent was removed by passage through a centrifuge gel filtration column, and the eluate was diluted into 50 mM $Na_2B_4O_7$, 5 mM $MgSO_4$, and 10% (v/v) methanol, pH 9.1, to a final protein concentration of 100 μg/mL. This solution was left at 35 °C for 1 h. Initially most of the ATPase activity could be restored by addition of 1 mM dithiothreitol to the assay, but after 1-h incubation, no activation occurred after addition of dithiothreitol. This suggests that the ATPase is now labeled with the nitrobenzofuran group attached to an amino group (Ferguson et al., 1975). At this stage, [^{3}H]o-ATP (125 Ci/mol) was added to a concentration of 5 μM, and the incorporation of radiolabel was followed.

None of these pretreatments affected the rate of incorporation of radiolabeled o-ATP into the ATPase.

Spectroscopy. ^{1}H NMR spectra were obtained at 360 MHz by using a Bruker WH360 spectrometer. The sample temperature was 25 °C, and the sample concentration was 4–8 mg/mL. Mass spectra were obtained by electron impact with a direct insertion probe on an MS-30 mass spectrometer.

Preparation of o-ATP and Borohydride-Reduced o-ATP. o-ATP and borohydride-reduced o-ATP were prepared as described by Lowe & Beechey (1982). Radiolabeled o-ATP was prepared in a similar manner to unlabeled o-ATP, but a smaller Sephadex G-10 column (0.4 × 17 cm) was used, with a flow rate of 1 mL/h. The radiochemical purity was determined by thin-layer chromatography (TLC) on poly(ethylenimine)–cellulose plates eluted with 1 M LiCl as described by Lowe & Beechey (1982). Samples of either o-ATP or radiolabeled o-ATP, or o-ATP pretreated for 10 min with a 5-fold molar excess of $NaBH_4$, showed a purity of greater than 95% on analysis by TLC. The concentration of adenine nucleotides was estimated by measurements of the A_{260} of aqueous solutions at pH 7. An extinction coefficient of 15000 $L \cdot mol^{-1} \cdot cm^{-1}$ was assumed. Since an additional chromophore is present in the adenine-containing elimination product (compound II, Scheme I), the concentrations of this compound were slightly overestimated.

Preparation of Compound II. A solution containing 144 mg (0.23 mmol) of o-ATP, 50 mM $Na_2B_4O_7$, and 5 mM $MgSO_4$ at pH 9.1 in a volume of 5 mL was left at 35 °C for 22 h. The mixture was left at 4 °C for a further 3 h, and the white inorganic precipitate produced was removed by centrifugation. A portion of the supernatant containing 43 μmol of 260-nm absorbing material was diluted to 10 mL with water and was applied to a column of DEAE-Sephadex A-25 (chloride form, 0.9 × 8 cm). The column was eluted with water, producing a yield of 35 μmol of compound II. This product showed only a single UV-absorbing spot on TLC: ^{1}H NMR ([$^2H_6$]dimethyl sulfoxide) δ 9.35 (s, 1 H, 3′-CHO), 8.46 (s, 1 H, 8-H), 8.37 (s, 1 H, 2-H), 7.73 (br, 3.6 H, 6-NH_2, 2′-OH, 2″-OH), 6.13 (d, J = 5.2 Hz, 1 H, 1′-H), 5.89 (d, J = 3.3 Hz, 1 H, 5″-H), 5.59 (dd, J = 5.2 and 1.2 Hz, 1 H, 2′-H), 5.56 (d, J = 3.3 Hz, 1 H, 5′-H). The chemical shifts are referred to the residual signal of [$^2H_5$]dimethyl sulfoxide, which was assigned the value of 2.615 dpm.

The resonance at δ 7.73 disappeared after shaking with ^2H_2O. The fine coupling on the resonance at δ 5.59 was also removed. The rest of the spectrum was unchanged, save for small changes in the chemical shifts. These were less than 0.1 ppm.

Preparation of Compound III. o-ATP (300 μmol, 300 mg) was dissolved in 6.9 mL of a solution of 50 mM $Na_2B_4O_7$ and 5 mM $MgSO_4$, pH 9.1, and left at 40 °C for 18 h. The mixture was then left for a further 2 h at 4 °C. The precipitate which formed was removed by centrifugation and washed with water. The supernatant and the washings were combined. TLC showed only two adenine-containing compounds. These appeared to be unreacted o-ATP (20%) and compound II (80%). An aqueous solution of 1.58 M sodium borohydride (1.67 mL) was added in 100-μL aliquots over a period of 1 h at room temperature. After a further hour, 9 M H_2SO_4 was added to bring to pH 7. The mixture was diluted to 25 mL and applied to a column of DEAE-Sephadex A-25 (0.9 × 7.3 cm; equilibrated with a solution containing 0.5 M NaCl/1 mM HCl and washed thoroughly with water). The column was eluted with water, and the first 35 mL of eluate was pooled and lyophilized. The solid was extracted with 4 × 40 mL of ethanol. The ethanolic extract was taken to dryness on the rotary evaporatory. The residue was dissolved in water and lyophilized, yield 76.7 mg, 213 mmol.

TLC analysis of this product on silica gel with acetonitrile/water (4:1 v/v) as eluant showed three adenine-containing components. Approximately 85% of the material was compound III. Further purification was carried out by preparative TLC using the same eluant. Material with an R_f of about 0.5 was eluted from the plate by repeated extraction with ethanol. This material which was chromatographically pure was used for ^{1}H NMR and mass spectral studies.

Mass spectrum of compound III showed m/e 252 (0.5, MH^+), 251 (0.5, M^+), 234 (4, $M^+ - OH$), 221 (4.5, $MH^+ - CH_2OH$), 220 (37, $M^+ - CH_2OH$), 208 (5), 207 (4), 179 [26, $MH^+C(CH_2)(O) - CH_2OH$], 178 [100, $M^+ - C(CH_2)(O) - CH_2OH$], 161 (12, 178 − OH), 149 (69, $AHCH_2^+$), 148 (74, $AHCH^+$), 136 (29, AH_2^+), 135 (66, AH^+), 108 (36, $AH^+ - HCN$), 81 (10, 108 − HCN), 67 (18), 57 (19), 55 (11), 54 (2, 81 − HCN), 53 (12), 43 (45, H_3CN_2), 42 (10). The values in parentheses are the percentage intensities of peaks in relation to the most intense (100%) in the mass spectrum. M^+ refers to the molecular ion; A is the adenine moiety, $C_5N_5H_4$. Only peaks with a relative intensity greater than 10% are listed, except for values of m/e greater than 200.

^{1}H NMR spectrum ([$^2H_6$]dimethyl sulfoxide) gave δ 8.37 (s, 1 H, H-8), 8.28 (s, 1 H, H-2), 7.38 (s, br, 2 H, 6-NH_2), 6.30 (t, J = 5.4 Hz, 1 H, 1′-H), 5.47 (t, J = 6.3 Hz, 1 H, 2′-OH), 5.18 (t, J = 5.8 Hz, 1 H, 3′-OH), 4.40 (d, J = 2 Hz, 1 H, 5″-H), 4.29 (dd, J = 2 and 0.9 Hz, 1 H, 5′-H), 4.03 (dd, 2 H, 2′-H, 2″-H), 3.89 (ABX system, 2 H, 3′-H, 3″-H). Irradiation of either resonance at δ 6.30 or δ 5.47 led to the collapse of the double doublet at δ 4.03 to a single doublet. Irradiation of the triplet at δ 5.18 collapsed the multiplet at δ 3.89 to an AB quartet. Irradiation of the multiplet at δ 3.89 collapsed the triplet at δ 5.18 to a singlet and the double doublet at δ 4.29 to a single doublet. The signals at δ 7.38, 5.47, and 5.18 disappeared and the signal at δ 4.03 collapsed to a doublet, J = 5.4 Hz, after shaking with ^2H_2O. The multiplet at δ 3.89 formed two doublets at δ 3.94 and 3.86 coupled to each other, J = 14.7 Hz.

The spectrum may be compared with those of other enofuranosyl nucleosides (Prisbe et al., 1976). The vinyl protons of 9-(5-deoxy-β-D-*erythro*-pent-4-enofuranosyl)adenine resonate at 4.21 and 4.32 ppm. The geminal coupling constant is 2 Hz. The anomeric proton resonates at 6.17 ppm, while the H-2′ and H-3′ protons resonate at 4.85 and 4.73 ppm, respectively. The 4-bond coupling constant $J_{3'-5'}$ was 0.5 Hz, and the coupling constant J_{H-C-OH} was 5 Hz.

Results

Reversible Interactions between o-ATP and the ATPase. In the presence of Mg^{2+} ions, o-ATP is a substrate for the

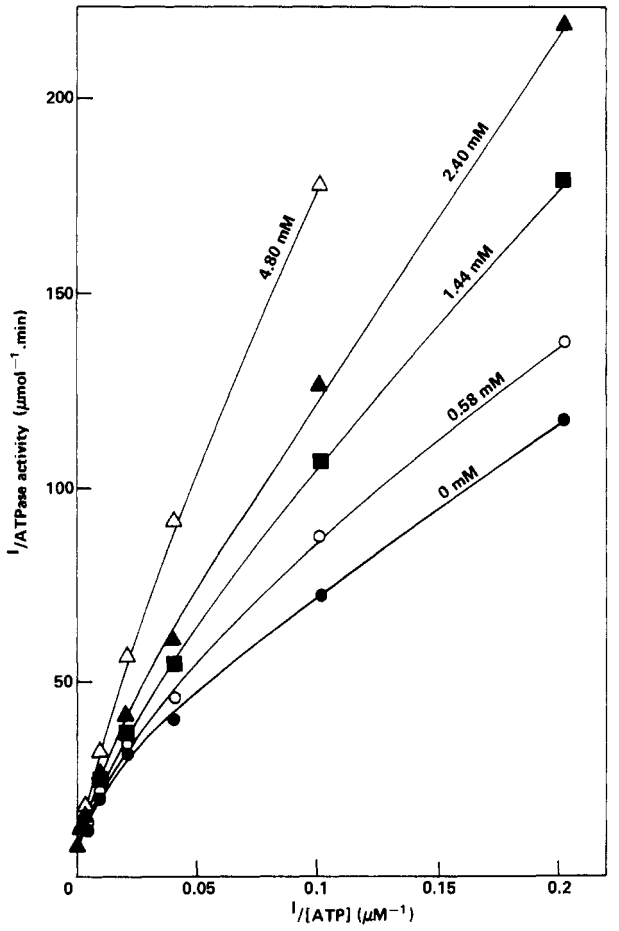


FIGURE 1: Reversible inhibition of ATPase activity by o-ATP. ATPase was dissolved in 1 mL of the standard ATPase assay medium at 30 °C (final concentration 5 μg of protein/mL). ATP was added to give final concentrations of 4.9, 9.8, 24.6, 49, 98, 246, 492, 983, and 2460 μM ATP. o-ATP was then added, within 15 s, to give final concentrations of 0.58, 1.44, 2.40, and 4.80 mM o-ATP. The rates of ATPase activity were measured within 3 min after the additon of the o-ATP. The numbers on the curves refer to the concentration of o-ATP in the assays.

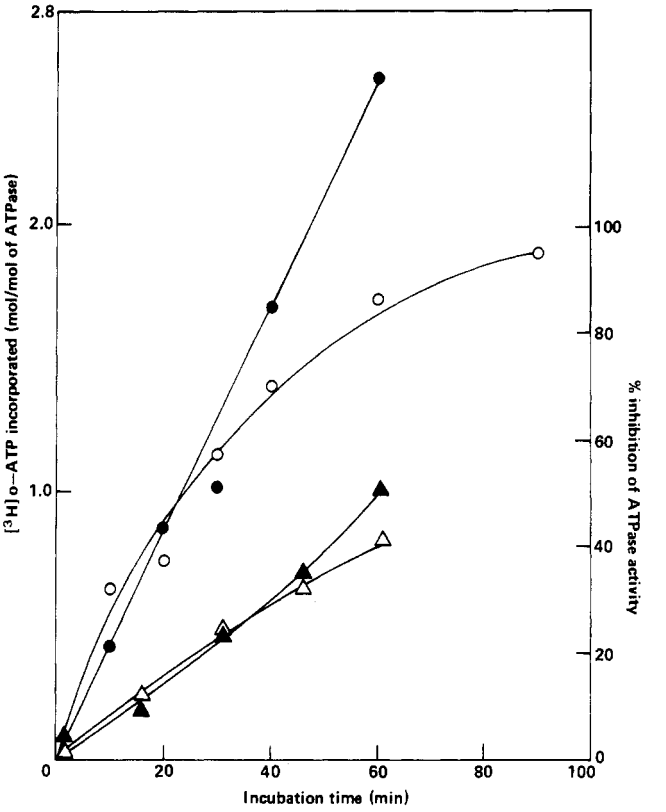


FIGURE 2: Rates of incorporation of o-ATP into the ATPase and the concurrent inhibition of ATPase activity. ATPase (100 μg of protein/mL) was incubated with either 9.4 or 2.5 μM [^{3}H]o-ATP (460 and 380 Ci/mol, respectively) at 35 °C in 50 mM $Na_2B_4O_7$, 5 mM $MgSO_4$, and 10% (v/v) methanol, pH 9.1. Aliquots of 20 μL were removed for ATPase assays in the standard assay medium. To further aliquots of 100 μL were added 25 μL of BSA (40 mg/mL) and 100 μL of 10% (v/v) $HClO_4$. The precipitate was collected by centrifugation and washed twice by resuspension in 5% (v/v) $HClO_4$. It was then dissolved by warming in a solution containing 100 mM Tris and 1% (w/v) $NaDodSO_4$. The radioactive content was then measured. The incorporation of radiolabel was calculated by assuming quantitative precipitation of the ATPase protein. Incorporation of o-ATP: (●) 9.4 μM o-ATP; (▲) 2.5 μM o-ATP. Inhibition of ATPase activity: (○) 9.4 μM o-ATP; (△) 2.5 μM o-ATP.

chloroform-released mitochondrial ATPase (Lowe et al., 1979b). MgADP and MgATP decreased the rate of hydrolysis of o-ATP. These results suggest that o-ATP interacts at a catalytic site.

o-ATP can act as a reversible inhibitor of ATP hydrolysis, although on prolonged incubation with the enzyme irreversible inhibition ensues. Since nonlinear kinetics were obeyed by the ATPase (Figure 1), a detailed analysis of the mechanism could not be made. A considerable competitive element in the inhibition was apparent however, since at high concentrations of ATP this inhibition by o-ATP can be relieved. This again suggests the interaction of o-ATP at a catalytic site.

Irreversible Interactions between o-ATP and the ATPase. o-ATP was incubated with the ATPase, and samples were removed at intervals for assay of ATPase activity. Under these conditions, the inhibition induced by o-ATP was apparently irreversible since the ATPase remained inhibited after extensive dilution, gel filtration, or precipitation wth ammonium sulfate. When the inhibited enzyme was diluted into the assay medium, ATP could not reverse the inhibition within the time period of the assay.

Preincubation of the ATPase with $NaBH_4$-reduced o-ATP did not inhibit the enzyme activity, suggesting that the aldehyde groups on o-ATP are involved in the inhibition. $NaBH_4$ treatment of the ATPase irreversibly inhibited by o-ATP did not affect the inhibition.

The kinetics of inhibition by o-ATP are pseudo first order with respect to the remaining enzyme activity, provided that there is an excess of o-ATP over the ATPase (Lowe et al., 1979b). At pH 7, the rate of inhibition by 1 mM o-ATP was 40-fold less than that at pH 9. The kinetic data can be explained in terms of a two-step mechanism where o-ATP reacts reversibly with the ATPase molecule to form an inhibitor/enzyme complex followed by an irreversible step (Kitz & Wilson, 1962), with K_m = 740 μM and k_2 = 0.13 min^{-1} at pH 8.0 and K_m = 55 μM and k_2 = 0.18 min^{-1} at pH 9.1.

Covalent Incorporation of Radiolabeled o-ATP. ATPase was incubated with radiolabeled o-ATP, and at intervals, samples were removed, and the amount of radiolabel covalently bound was estimated (see Experimental Procedures). ATPase which has been 50% inhibited by incubation with [^{3}H]o-ATP has 1.05 mol of [^{3}H]o-ATP covalently bound per mol of ATPase, whereas at 95% inhibition the incorporation has increased to 6.8 mol/mol of ATPase. The extent of incorporation of [^{3}H]o-ATP correlates well with the inhibition of ATPase activity up to 60% inhibition [see Figure 2 and Lowe et al. (1979b)]. At higher levels of inhibition, the extent of inhibition is no longer directly proportional to the amount of o-ATP bound to the enzyme molecule. The rate of inhibition

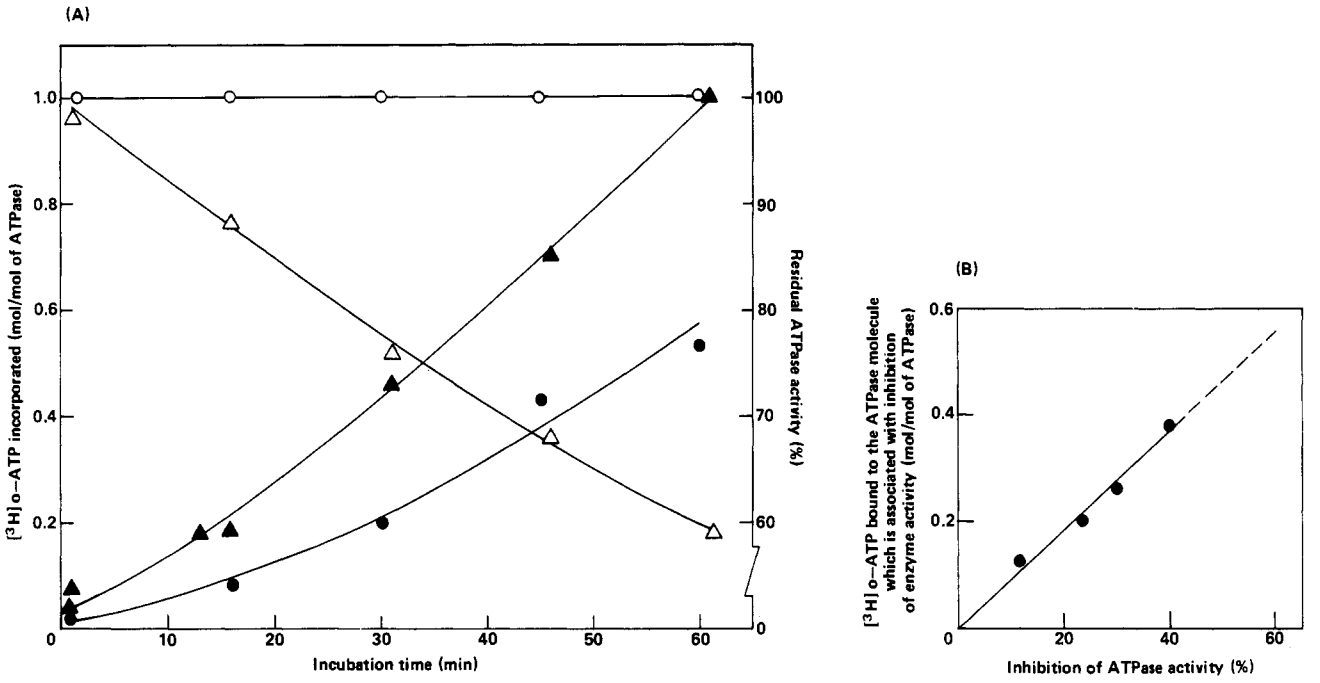


FIGURE 3: (A) Time course of the incorporation of o-ATP into the ATPase molecule in the presence and absence of ADP. ATPase (100 μg of protein/mL) dissolved in 50 mM $Na_2B_4O_7$, $MgSO_4$, and 10% (v/v) methanol, pH 9.1, was incubated with 2.5 μM [^{3}H]o-ATP (380 Ci/mol) at 35 °C in the presence or absence of 9.3 mM ADP. Aliquots of 20 μL were removed for ATPase assay in the standard assay medium. The covalent binding of o-ATP to the ATPase was estimated by the perchloric acid precipitation method in the presence of BSA. Incorporation of o-ATP into the ATPase: (●) ADP present; (▲) ADP absent. The ATPase activity is presented as a percentage of the activity of the ATPase incubated with neither o-ATP nor ADP: (○) ADP present; (△) ADP absent. (B) The relationship between the inhibition of ATPase activity by o-ATP and the reduction of the incorporation of o-ATP caused by ADP. The differences in the incorporation of [^{3}H]o-ATP into the ATPase incubated with and without ADP are plotted against the inhibition of ATPase activity by o-ATP in the absence of ADP.

induced by o-ATP decreases with time (pseudo-first-order kinetics) whereas the rate of incorporation of radiolabel stays constant or even has a tendancy to increase (Figure 2). These results suggest that with longer incubation times an increased amount of nonspecific labeling occurs. At low levels of incorporation of o-ATP into the ATPase molecule, the inhibitor appears to bind to two specific sites.

Effects of ADP and ATP on the Interactions between o-ATP and the ATPase. The results presented in Figure 3A show that although MgADP can completely prevent the inhibition of ATPase activity by o-ATP, radiolabeled o-ATP was still incorporated into the ATPase. The extent of the labeling was reduced in comparison to an incubation of o-ATP with ATPase in the absence of ADP. These results indicate that o-ADP can bind at the o-ATP inhibitory site. In the experiment shown in Figure 3, after 60-min incubation of the ATPase with o-ATP in the absence of MgADP, the ATPase activity was 40% inhibited, and approximately 1 mol of o-ATP was bound covalently per mol of ATPase. The activity of the ATPase preparation incubated with both o-ATP and MgADP was not inhibited and showed an incorporation of 0.55 mol of o-ATP/mol of ATPase. The degree of protection exerted by ADP can be seen more clearly from Figure 3B, where the difference in the extent of incorporation of o-ATP into the ATPase between the incubations with and without ADP is plotted against the inhibition of ATPase activity induced by o-ATP in the absence of ADP. The incorporation of radiolabel displayed on this graph is thus that which is associated with the inhibition of ATPase activity. Extrapolation to 100% inhibition of ATPase activity shows that 0.95 mol of o-ATP is associated with complete inhibition. Since in the absence of ADP 2 mol of o-ATP is bound to the completely inhibited ATPase (see above), there must be a second site for o-ATP binding at which ADP does not bind and binding of o-ATP does not cause inhibition of ATPase activity.

ATP in the presence of Mg^{2+} also prevented inhibition of ATPase activity by o-ATP and also did not prevent completely the covalent incorporation of o-ATP into the ATPase molecule. The rate of incorporation of [^{3}H]o-ATP into the ATPase is lower in a medium containing 0.2 mM EDTA than in one containing 5 mM $MgSO_4$. Furthermore, EDTA behaved similarly to ADP (Figure 3) in that it prevented o-ATP from exerting its inhibitory action, suggesting that the binding of o-ATP as the site causing inhibition requires Mg^{2+}.

Effects of Other Ligands on the Binding of o-ATP. The rates and extent of covalent binding of o-ATP to the chloroform-released ATPase were not affected when the enzyme activity was inhibited by either adenylyl 5′-imidodiphosphate or efrapeptin or phenylglyoxal or 7-chloro-4-nitro-2,1,3-benzoxadiazole attached to an amino group (Ferguson et al., 1975).

Effects of o-ATP on the Fluorescence of the Aurovertin B/ATPase Complex. Chloroform-released ATPase (50 μg of protein/mL) was incubated with either 90 μM ATP or 90 μM o-ATP for 1 h at 30 °C in 50 mM $Na_2B_4O_7$, 5 mM $MgSO_4$, and 10% (v/v) methanol, pH 9.1. At this stage, the ATPase activities were inhibited by 0 and 95%, respectively, relative to controls incubated without added nucleotides. The solutions were than placed in a fluorometer at 30 °C and titrated with aurovertin B. Both the ATP- and o-ATP-treated enzyme preparations showed a large enhancement of aurovertin fluorescence. The maximum enhancement of fluorescence was about 30% less than that observed with ATPase untreated with ATP or o-ATP. This experiment shows that aurovertin can still bind to its "fluorescent binding site" when the enzyme is inhibited by prior incubation with o-ATP. In a second experiment, the addition of ATP did not quench the fluorescence of the complex of aurovertin B and the o-ATP-treated ATPase. This suggests that o-ATP binds to the ATP site which causes quenching of aurovertin fluorescence.

Localization of the Bound o-ATP on the ATPase. ATPase was preincubated with radiolabeled o-ATP to inhibit the enzyme activity. The distribution of the radiolabel among the subunits was then examined by $NaDodSO_4$–polyacrylamide gel electrophoresis. No cross-linked species were observed, showing that the dialdehyde groups do not interact with more than one subunit simultaneously. ATPase inhibited to 50% by incubation with [^{3}H]o-ATP contained 1.05 mol of o-ATP bound per mol of ATPase, of which 0.7–0.9 mol/mol of ATPase was associated with the α and β subunits, distributed equally between the two, and 0.1–0.2 mol with the γ subunit.

From the data presented in Figure 3A, it can be seen that the radiolabel incorporated in the presence of ADP would not be expected to be associated with the inhibitory effects of o-ATP. The distribution of radiolabel among the ATPase subunits was therefore investigated in ATPase which had been preincubated with [^{3}H]o-ATP either in the presence or in the absence of ADP. There was a definite, if small, difference in the distribution of radioactivity between the α/β and γ subunits. ADP appeared to preferentially prevent incorporation of o-ATP into the α and β subunits. The ratio of the radiolabel associated with the α and β subunits to that associated with the γ subunit fell from 8.5 in the o-ATP-treated preparation to 5.6 when ADP was present. The distribution of radiolabel between the α and β subunits was not affected by the presence of ADP in the incubation.

It can be seen from the data presented in Figure 3B that of the incorporated radiolabel from o-ATP, 1 mol/mol of ATPase is responsible for the inhibition of ATPase activity. Of this, 92% is bound to the α/β subunits and 8% to the γ subunit. The simplest explanation for this result is that the small binding to the γ subunit is of a nonspecific nature, whereas the incorporation of o-ATP into the α/β subunits causes the observed inhibition of the ATPase activity. It is less clear whether the localization of noninhibitory, bound o-ATP is specific to the α/β subunits or whether it is simply binding in proportion to the amount of protein present.

β-Elimination Reaction Undergone by o-ATP. When o-ATP is kept at room temperature at pH 7, a new adenine-containing compound (compound II, Scheme I) is produced. This reaction occurs very much more rapidly at elevated temperatures and at alkaline pH. In 50 mM $Na_2B_4O_7$, pH 9.1 at 30 °C, 50% decomposition of o-ATP occurred after 5 h. After 24 h, the extent of decomposition was 83%. During this process, no orthophosphate was produced, and only one adenine-containing product was formed. Compound II was isolated (see Experimental Procedures), and it contained no phosphorus. It appeared to contain at least one aldehyde group since (a) it remained at the origin of poly(ethylenimine)–cellulose TLC plates eluted with LiCl solutions, unless it was first reduced with $NaBH_4$, (b) it was detectable on silica gel TLC plates by formation of a yellow color with 2,4-dinitrophenylhydrazine, and (c) its ^{1}H NMR spectrum showed a resonance at 9.35 ppm, typical of an aldehydic proton. The UV spectrum of compound II, recorded in aqueous solution at pH 7, differed from that of ATP (or o-ATP) in that it had λ_{max} = 257 nm [λ_{max}(ATP) = λ_{max}(o-ATP) = 259 nm] and λ_{min} = 225 nm [λ_{min}(ATP) = λ_{min}(o-ATP) = 227 nm]. A difference spectrum, compound II minus ATP, revealed two peaks at 235 nm (ϵ = 3700) and 280 nm (ϵ = 350). These could be attributable to the presence of an α,β-unsaturated group (Graselli, 1973). The ^{1}H NMR spectrum is consistent with structure II (Scheme I). It is interesting that the C-3′ aldehyde group exists as a free structure whereas the C-2′ aldehyde group is a hydrate. It appears that the electron-withdrawing groups on C-1′ promote hydration [cf. CH_3CHO vs. $CCl_3CH(OH)_2$], whereas the conjugation with the C-4′–C-5′ double bond reduces the tendency of the C-3′ aldehyde to hydrate.

Confirmation of the proposed structure comes from an examination of its borohydride-reduced derivative (compound III, Scheme I). This compound has a UV spectrum identical with that of ATP and does not contain aldehyde groups. Its ^{1}H NMR and mass spectra confirm its structure to be that shown in Scheme I, structure II.

Since compound II did not contain phosphorus, we investigated the fate of the triphosphate group after alkaline degradation of o-ATP. TLC on poly(ethylenimine)–cellulose plates eluted with 1 M LiCl showed that a phosphorus-containing compound was present. The R_f value of about 0.05 was identical with that of authentic tripolyphosphate. The mobility was not changed by prior reduction of the sample with $NaBH_4$, showing that the triphosphate moiety was not attached to an aldehyde group. Schwartz & Gilham (1972) have observed a similar degradation reaction occurring during the alkaline treatment of periodate-oxidized AMP, although the products were not directly characterized. It is thought that the mechanism of the decomposition reaction which o-ATP undergoes involves β elimination of the triphosphate group, leaving compound II as depicted in Scheme I.

Involvement of an Elimination Reaction in the Incorporation of o-ATP into ATPase. A mixture of [α-^{32}P]o-ATP and [2,8-$^3H_2$]o-ATP was incubated with the ATPase until the enzyme activity was inhibited by 65%. After borohydride reduction, the mixture was gel filtered (see Figure 4). The ATPase activity eluted in the void volume and was associated with 1.50 ± 0.29 mol of tritium/mol of ATPase and only 0.17 ± 0.17 mol of ^{32}P/mol of ATPase. The peaks of radioactivity due to phosphorus and to tritium eluting with the void volume occurred at the same fraction. This experimental result shows that when o-ATP is bound to the ATPase, most of the triphosphate group has been lost. The radiolabel eluting after the ATPase appeared as two peaks (Figure 4), the radioactive phosphorus being eluted prior to the tritium. An explanation is that a significant proportion of the phosphate is in the form of the tripolyphosphate ion whereas the tritium peak is mainly borohydride-reduced o-ATP. Since adenine-containing nucleotides are retarded on Sephadex columns (Bernofsky, 1975; Engel, 1977), the tritium elutes after the radioactive phosphorus.

It would appear that under the experimental conditions used most of the elimination reaction occurs after the binding of o-ATP to the ATPase. The inhibition induced by incubation of o-ATP with the ATPase cannot be due solely to compound II produced by degradation of the o-ATP as can be seen from the following analysis. The loss of ATPase activity induced by 5 μM o-ATP shows a half-time of about 36 min. It can be calculated from Figure 5 that 2 μM compound II leads to inhibition with a half-time of 36 min (see following section). Thus, if the inhibition induced by o-ATP is due solely to the action of compound II, the concentration of compound II must be greater than 2 μM. The initial amount of compound II in 5 μM o-ATP is less than 0.3 μM (as shown by TLC analysis), and since the half-time for decomposition of o-ATP to compound II is approximately 300 min, it would take over 2 h to achieve a concentration of compound II greater than 2 μM. Thus, the major cause of inhibition is not compound II produced by degradation of o-ATP. Also, after incubation of o-ATP with ATPase under conditions similar to those described in Figure 4, about 25% of the o-ATP had undergone

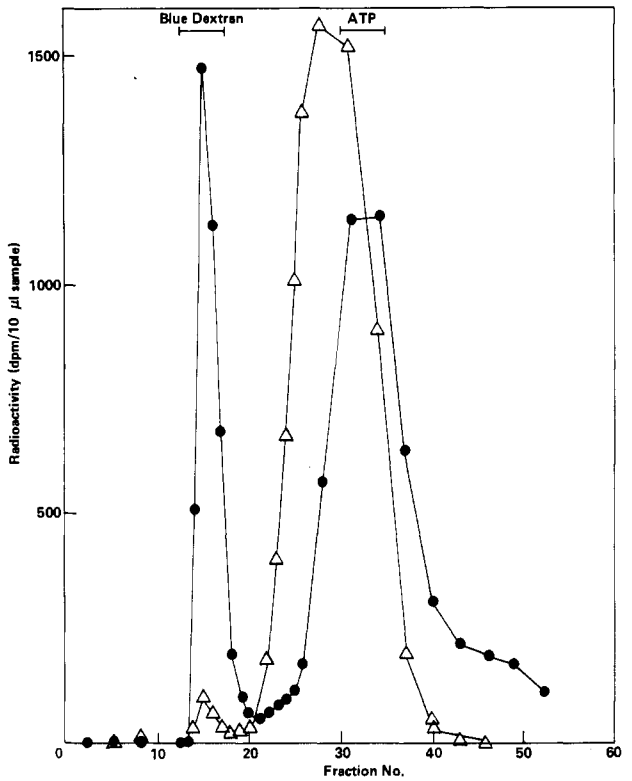


FIGURE 4: Radiolabel associated with the ATPase after treatment with a mixture of [α-^{32}P]o-ATP and [^{3}H]o-ATP. ATPase (200 μg of protein in 200 μL) was incubated with 17.4 μM o-ATP (a mixture of [α-^{32}P]- and [^{3}H]o-ATP) at 30 °C in 50 mM $Na_2B_4O_7$, 5 mM $MgSO_4$, and 10% (v/v) methanol, pH 9.1. The final specific activity of [^{32}P]ATP was 216 Ci/mol, and that of [^{3}H]o-ATP was 173 Ci/mol. After incubation for 125 min, the mixture was chromatographed on a column of Sephadex G-25 (26.5 × 0.7 cm) equilibrated with 50 mM Tris/H_2SO_4, 60 mM K_2SO_4, and 1 mM EDTA, pH 8.0. Fractions of 0.4 mL were collected and assayed for radiolabel. The elution profiles of ^{3}H (●) and ^{32}P (△) are shown. In a separate experiment, the elution positions of Blue Dextran and ATP were determined. These are marked by the bars above the elution profile for the radioactivity. The ATPase activity eluted at the same position as the Blue Dextran.

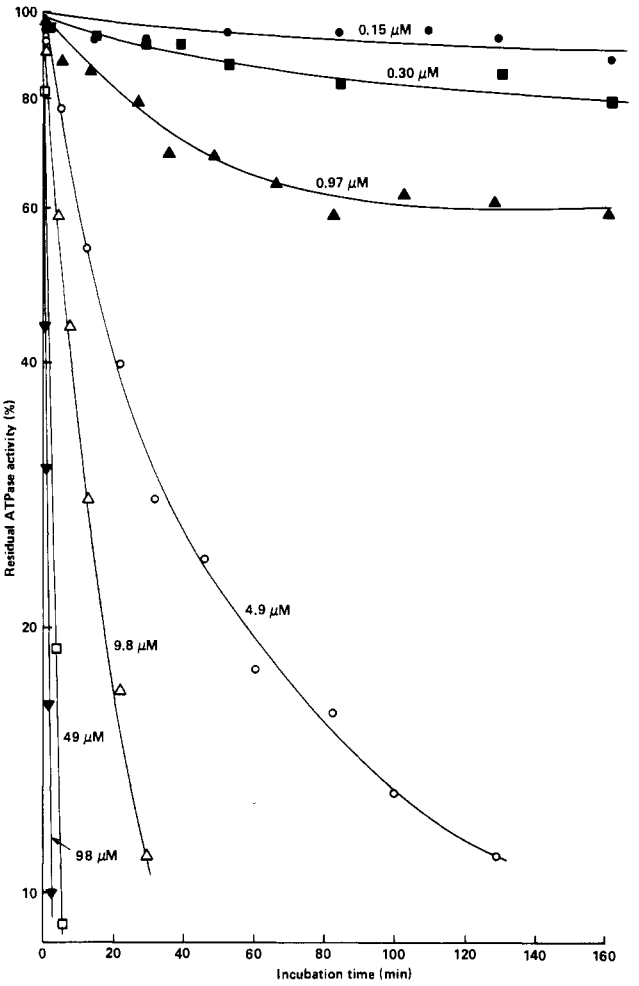


FIGURE 5: Inhibition of ATPase activity by compound II. ATPase (100 Mg of protein/mL) was incubated in 50 mM $Na_2B_4O_7$, 5 mM $MgSO_4$, and 10% (v/v) methanol, pH 9.1 at 35 °C, for 2 h. Compound II was added to give the final concentrations shown, and the incubation was continued at 35 °C. Aliquots of 20 μL were removed at intervals for assay of ATPase activity by dilution into the standard assay medium. The ATPase activity is expressed as a percentage of a control to which no compound II was added.

an elimination reaction to produce compound II in free solution. However, we have shown that 60–90% of the enzyme-bound o-ATP has undergone elimination. Thus, it would seem likely that the elimination reaction occurs faster on the enzyme than in free solution.

Comparison of the Inhibitory Properties of Compound II and o-ATP. Compound II retains a dialdehyde grouping, and chemically this compound would be expected to be as reactive as o-ATP. Incubation of compound II with the ATPase results in a time-dependent complete irreversible inhibition of ATPase activity. Pseudo-first-order kinetics of inhibition were obeyed provided that the excess of compound II over the ATPase was at least 30-fold (Figure 5). This behavior differs from that observed with o-ATP, where the inhibition was pseudo first order with only a small excess of inhibitor over the ATPase (Lowe et al., 1979b). The inhibition induced by these two compounds differs further in that with equal concentrations of inhibitor the rate of inhibition exerted by compound II is greater than that observed with o-ATP. Thus, at an ATPase concentration of 100 μg of protein/mL, 5 μM o-ATP caused inhibition with a half-time of approximately 36 min, whereas that with 5 mM compound II was 15 min. The initial rates of inhibition were used to determine whether a rate-saturation effect could be observed with increasing concentrations of compound II. The plot of half-time for inhibition at pH 9 against the reciprocal of the inhibitor concentration was straight and passed very close to the origin, i.e., $K_m > 1$ mM, $k_2 = 10$ min^{-1}. A rate-saturating effect was not clearly seen, demonstrating that the irreversible step occurs very quickly relative to the binding step [mechanism described in Kitz & Wilson (1962)]. In contrast, with o-ATP, a clear rate-saturating effect was visible, $K_m = 55$ μM and $k_2 = 0.17$ min^{-1}.

The high value of the K_m for the irreversible inhibition exerted by compound II suggests that it has little specificity for its inhibitory binding site, presumably because it does not possess a triphosphate group. The non-pseudo-first-order kinetics (Figure 5) could be explained if sufficient compound II reacts with the ATPase so as to significantly change the concentration of compound II in solution. This would again suggest multiple binding sites for compound II on the enzyme.

The fast rate of inhibition exerted by compound II is not related to its lack of specifity. o-Adenosine and o-CTP inhibit the ATPase activity very slowly. The half-times for inhibition were 495 and 123 min, respectively, with 5 μM o-adenosine and o-CTP. These compounds would not be expected to bind to the nucleotide binding sites on the enzyme and thus would be expected to exert their inhibitory effects nonspecifically. In contrast, o-ADP and o-ATP inhibit much faster, $t_{1/2} = 36$ min (5 μM nucleotide), although still more slowly than compound II. It is thought that compound II inhibits ATPase

activity more rapidly than o-ATP because the rate-determining step in the inhibition mechanism is different (see Discussion).

Discussion

The results presented here show that o-ATP is a time-dependent irreversible inhibitor which becomes covalently bound to the chloroform-released mitochondrial ATPase. Evidence that o-ATP binds at specific sites is that at low levels of inhibition, incorporation of o-ATP is directly proportional to the loss of enzyme activity (Figure 2). Furthermore, the rate of irreversible inhibition reaches a maximum value with increasing concentrations of the inhibitor. The concentrations of o-ATP that are required for half-maximal inhibition are low, 55 μM at pH 9, suggesting that binding of o-ATP at specific sites occurs prior to the irreversible binding step. Evidence that o-ATP interacts with a catalytically active site is that o-ATP acts as a substrate in the presence of Mg^{2+} and as a competitive inhibitor of ATP hydrolysis (Figure 1). The irreversible inhibition exerted by o-ATP is dependent upon Mg^{2+}, which is also needed for the interaction of ATP at a catalytic site. Also, the substrate, MgATP, and the product, MgADP, prevent the inhibition induced by o-ATP. Prebound o-ATP prevents the ATP-induced quenching of aurovertin fluorescence, implying that o-ATP may bind to the ATP-quenching site, which in turn may be related to the catalytic site (Ferguson et al., 1976).

The covalent modification of an amino group on the ATPase by 7-chloro-4-nitro-2,1,3-benzoxadiazole does not affect the incorporation of o-ATP into the ATPase, suggesting that this amino group is not essential for the incorporation of o-ATP. Since o-ATP binds to an amino group (see below), at least two amino groups essential for ATPase activity must be present on the enzyme.

The covalently bound o-ATP is thought to be the cause of the observed inhibition of ATPase activity since (a) removal of noncovalently bound o-ATP by gel filtration in a high ionic strength buffer does not change the extent of the inhibition, (b) a good correlation exists between the extent of incorporation of o-ATP and the induced inhibition, and (c) the maximum rate of inhibition occurs at alkaline pH, which favors the reaction between o-ATP and amino groups (Lowe et al., 1979a). At alkaline pH, however, the exchange of nucleotides into high-affinity sites is slow (Harris, 1978).

The results presented in Figures 2 and 3 suggest that there may be three types of covalent binding sites for o-ATP on the ATPase molecule. At one site (1 mol/mol of ATPase), the binding of o-ATP is prevented by ADP or ATP. When o-ATP is bound to this site, the ATPase activity is lost completely. This is likely to be a nucleotide binding site and could be a catalytic site. Of the o-ATP bound to this site, 85% is associated with the α/β subunits. o-ATP can bind at a second site (1 mol/mol of ATPase) at a rate similar to that of the first site binding. The binding of o-ATP to this site is not prevented by ADP or ATP, and the binding does not cause inhibition of ATPase activity. The nature of this site is not known, but it could represent reactive lysine residues on the α and β subunits. o-ATP also binds at a number of nonspecific sites at a lower rate so that after longer periods of time large amounts of o-ATP can be incorporated into the ATPase molecule. This could be due to the production of the adenine-containing elimination product (compound II) during prolonged incubation with o-ATP.

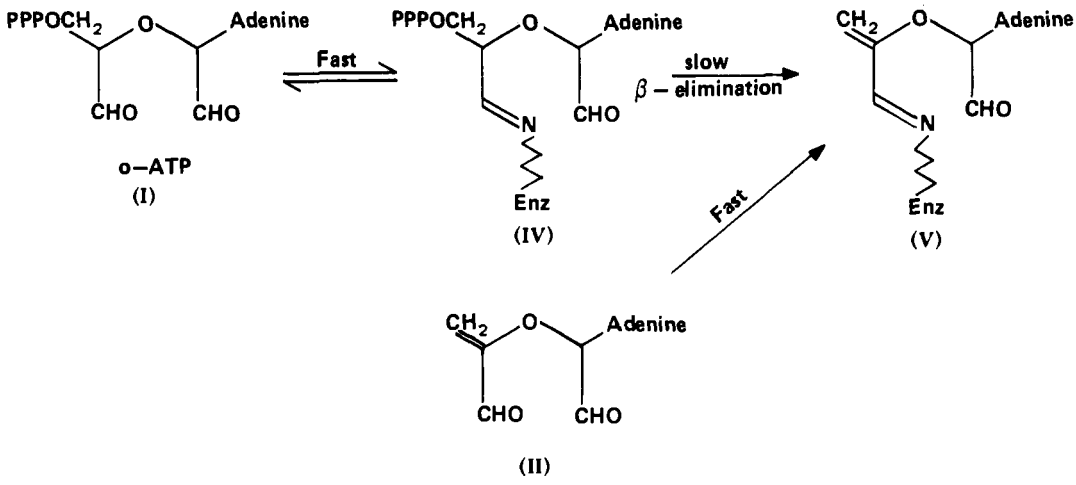
The inhibition of ATPase activity appears to occur if o-ATP binds to either the α or β subunit. Similar results were obtained by Lunardi et al. (1977) using a ribose-modified ATP analogue and by Slater et al. (1979) using 8-azido-ATP in the presence of Mg^{2+}. In other studies, nucleotide analogues were incorporated with different stoichiometries, and the labeling was specific for either the α or β subunit (see the introduction for references). These results could be rationalized by a consideration of the relative sizes of an ATP binding site and an ATPase subunit. The ATP molecule is about 1.5 nm in length and would cover an appreciable area of an α or β subunit, which are about 3–5 nm in diameter. Different parts of an ATP molecule could be in proximity to different areas of one subunit or indeed to another subunit. Thus, the subunit eventually labeled could vary with the location of the "affinity label" within the ATP molecule.

The results obtained on the location of the o-ATP inhibitory site on the ATPase could be interpreted in the following ways. (a) One site is present (on either the α or β subunit), but the ribose moiety is equally close to both subunits. (b) Two equivalent sites are present, one on an α and the other on a β subunit. Modification of either causes inhibition of ATPase activity. This model is consistent with the alternating catalytic site mechanism [reviewed in Penefsky (1979)].

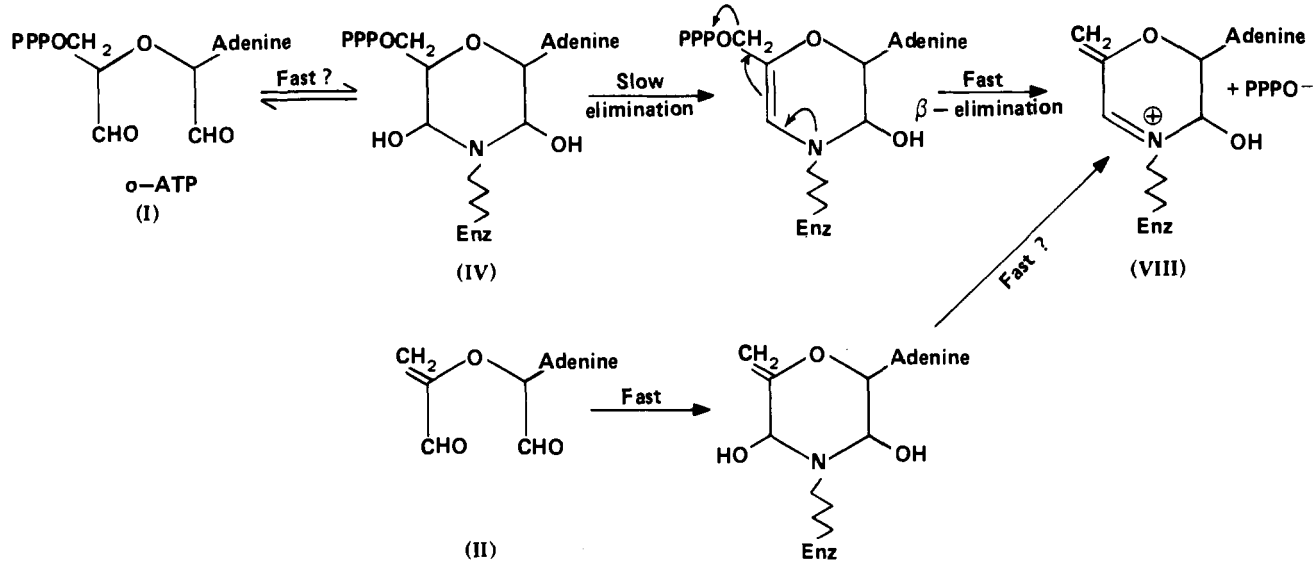
The interactions of o-ADP with the ATPases from *Mycobacterium phlei* (Kumar et al., 1979), *Escherichia coli* (Bragg & Hou, 1980; Bragg et al., 1981), pig heart (Baubichon et al., 1981), and ox heart (Kozlov & Milgrom, 1980) and of o-ATP with the ATPases from *M. phlei* (Kumar et al., 1979) and *E. coli* (Bragg et al., 1981) have been reported. Care must be taken in comparing and contrasting the previous results with the data reported here. Apart from the obvious differences in source and methods of preparation of the ATPases, all the experiments reported here were carried out in the presence of Mg^{2+}, i.e., conditions where one might expect interactions of o-ATP with the catalytic site. In contrast, in all of the previous studies, the binding of o-ATP and o-ADP has been examined in the absence of Mg^{2+}, where the enzyme is inactive.

Mechanism of Reaction of o-ATP and Compound II with the ATPase. It has been proposed that o-ATP reacts with proteins via a simple Schiff base with a lysine residue (Spoor et al., 1973; Easterbrook-Smith et al., 1976; Dallochio et al., 1976; Kotchetkov et al., 1977; Maccioni et al, 1979). In the case of its interaction with the ATPase, this cannot be the mechanism of reaction since the reaction products are produced irreversibly and are stable without borohydride reduction. We have shown that the aldehyde groups of o-ATP are essential for the inhibition and that the triphosphate group of o-ATP is not present in the o-ATP-inhibited ATPase. These data must be correlated with the following observations. (i) o-ATP only reacts with lysine residues among the amino acid residues found in proteins (Lowe et al., 1979a). (ii) In free solution, o-ATP undergoes an elimination reaction to produce compound II and liberate the tripolyphosphate ion. (iii) This reaction appears to occur at a faster rate when the o-ATP is bound to the ATPase molecule. Two schemes for the mechanism can be envisaged (Schemes II and III). In Scheme II, o-ATP binds reversibly to the enzyme, and a covalent complex (IV) is then formed by reaction of an aldehyde group of o-ATP with the amino group of a lysine residue to form a Schiff base. An elimination reaction occurs, and a triphosphate group is lost, leaving an enzyme-bound product containing a conjugated Schiff base grouping (V). Conjugated Schiff bases are more stable that simple Schiff bases (Monsan et al., 1975), and a stable link is formed without the necessity for reduction by borohydride. In this scheme, it is not obvious why the elimination reaction should occur faster on the enzyme than in free solution. In Scheme III, o-ATP reacts with the amino group of a lysine residue to produce a dihydroxymorpholine derivative

Scheme II



Scheme III



(VI). Elimination of water would then be followed by facile elimination of the tripolyphosphate anion to produce a quaternary ammonium compound (VII). This product would also be stable without reduction by borohydride. This mechanism has been proposed for the reactions between o-ATP and phosphofructokinase (Gregory & Kaiser, 1979) or phosphorylase kinase (King & Carlson, 1981). In this scheme, the lone pair of electrons on the nitrogen atom of the morpholine ring can aid the elimination process and hence the enzyme "catalyzes" the elimination reaction.

The observation that compound II inhibits ATPase activity faster than o-ATP can be explained by either of these schemes. In Scheme II, the slow elimination process is avoided. In Scheme III, a slightly different product could first be formed which avoids the slow elimination of water. A differentiation between these two mechanisms has to await further characterization of the enzyme-bound products.

Use of Periodate-Oxidized Nucleotides as Affinity Labels. The results presented here have several implications for the use of periodate-oxidized nucleotides as affinity labels. These compounds can decompose under mild conditions, and hence the purity should always be monitored. The adenine-containing elimination product compound II contains a dialdehyde grouping just as the starting compound, but it does not contain the phosphate grouping which might be essential for specific labeling of a nucleotide binding site. Thus, the elimination product is likely to bind to the protein with a low specificity. Moreover, this effect will be accentuated since the elimination product is more reactive than the parent compound. This is because it contains an unsaturated dialdehyde grouping which can form very stable adducts with amines [cf. the process of glutaraldehyde fixation (Monsan et al., 1975)]. The position of the radiolabel in the o-ATP molecule determines the apparent incorporation of o-ATP into the protein since the triphosphate group is lost. The stoichiometry of the binding of o-ATP cannot be ascertained by reduction of the enzyme/nucleotide complex by [^{3}H]borohydride since the reaction product is not yet known. Furthermore, periodate-oxidized nucleotides exist as mixtures of compounds in aqueous solutions (Lowe & Beechey, 1982), and the various isomers may bind differently to the enzyme molecule. Also, since periodate-oxidized nucleotides are bifunctional reagents, containing a dialdehyde grouping, cross-linking is possible, as has been seen with bovine serum albumin (Cysyk & Adamson, 1976).

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