An Active-Site-Directed Adenosine Triphosphate Analogue Binds to the β -Subunits of Factor F_1 Mitochondrial Adenosine Triphosphatase with its Triphosphate Moiety

By Valeriy L. DRUTSA,* Igor A. KOZLOV,† Yakov M. MILGROM,†
Zoya A. SHABAROVA* and Nadejda I. SOKOLOVA*

*Department of the Chemistry of Nucleic Acids and †Department of Bioenergetics, A. N. Belozersky Laboratory of Molecular Biology and Bio-organic Chemistry, Moscow State University, Moscow 117234, U.S.S.R.

(Received 3 May 1979)

The reaction of the mixed anhydride of [^3H]ATP and mesitylenecarboxylic acid and soluble mitochondrial adenosine triphosphatase is accompanied by the covalent binding of one molecule of the inhibitor to a molecule of the enzyme and results in the inhibition of adenosine triphosphatase activity by more than 90%. The electrophoresis of adenosine triphosphatase modified by reaction with the mixed anhydride of [^3H]ATP and mesitylenecarboxylic acid in polyacrylamide gel in the presence of sodium dodecyl sulphate showed that the inhibitor is bound to the β -subunit of the enzyme. The results suggest that ATP may also bind to the β -subunit of the adenosine triphosphatase with its triphosphate moiety.

The ATP synthesis in the oxidative phosphorylation system occurs in the active site of factor F_1 . It is a protein with a molecular weight of approximately 370000 and a complex subunit structure (for reviews see Racker, 1965; Senior, 1973; Pedersen, 1975; Kozlov & Skulachev, 1977). The subunit composition of a molecule of factor F_1 ($\alpha_3\beta_3\gamma\delta\epsilon$) suggested by Catterall & Pedersen (1971) is in good agreement with the data of Knowles & Penefsky (1972) on the molecular weights of the individual subunits [54000 (α), 51000 (β), 33000 (γ), 16000 (δ), 11000 (ϵ)] and factor F_1 as a whole.

The ATPase activity of isolated factor F_1 does not require minor subunits (γ , δ and ϵ). It was therefore suggested that the active (catalytic) site of mitochondrial ATPase is localized on the α - and/or β -subunits (Kozlov & Mikelsaar, 1974). Certain specific inhibitors of ATPase that covalently modify the protein molecule were used to determine the localization of the active site (Ferguson *et al.*, 1975; Lunardi *et al.*, 1977; Wagenvoort *et al.*, 1977; Budker *et al.*, 1977; Slater, 1978; Kemp, 1978; Esch & Allison, 1978). In the present paper the study of the localization of the active site of mitochondrial ATPase was carried out by using the mixed anhydride of [^3H]ATP and mesitylenecarboxylic acid. This inhibitor covalently modifies the active site of factor F_1 . Complete inhibition of ATPase is obtained as a result of the binding of one inhibitor residue per molecule of the enzyme (Kozlov *et al.*, 1977, 1979). The mixed anhydride of ATP and mesitylene-

carboxylic acid differs from a number of adenine nucleotide analogues used previously in studies of the localization of the active site of factor F_1 (Lunardi *et al.*, 1977; Wagenvoort *et al.*, 1977; Budker *et al.*, 1977; Slater, 1978; Kemp, 1978; Esch & Allison, 1978). The mixed anhydride of ATP and mesitylenecarboxylic acid modifies some nucleophilic group, located in the active site of factor F_1 at or near the site of binding of the γ -phosphate group of ATP (Kozlov *et al.*, 1977, 1979). Thus the use of the mixed anhydride of ATP and mesitylenecarboxylic acid may help to determine the localization of the group(s) of the active site that take(s) part in the acid-base catalysis of ATP hydrolysis. The results obtained in the present work indicate that this group(s) is localized on the β -subunit of factor F_1 .

Materials and Methods

Factor F_1 was isolated from bovine heart mitochondria by the method of Horstman & Racker (1970). The protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

The mixed anhydride of [^3H]ATP and mesitylenecarboxylic acid (150 Ci/mol) was synthesized from [$8\text{-}^3\text{H}$]ATP (15.4 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) as described elsewhere (Tretyakova *et al.*, 1978). The purity of the synthesized preparation of the mixed anhydride was checked chromatographically.

Modification of factor F_1 (3–4 mg/ml) was carried out in a medium containing 0.25 M-sucrose, 2 mM-EDTA, 20 mM-Mops/KOH buffer, pH 7.5, and 1 mM

Abbreviations used: ATPase, adenosine triphosphatase (EC 3.6.1.3); factor F_1 , soluble mitochondrial ATPase; Mops, 4-morpholinopropanesulphonic acid.

mixed anhydride of [^3H]ATP and mesitylenecarboxylic acid. The mixture was kept at room temperature for 60 min. The modified factor F_1 was separated from the excess of labelled ATP analogue by gel filtration through a column (0.8 cm $\times$ 20 cm) of Sephadex G-25 (fine grade).

Electrophoresis in 10% polyacrylamide gel in the presence of sodium dodecyl sulphate was performed as described by Weber & Osborn (1969). Some of the gels were stained with Amido Black, and the others were sliced into 1 mm-thick slices, which were placed in scintillation vials. After extraction of the radioactive material with 3 ml of 1.5% sodium dodecyl sulphate at 55–60°C, 17 ml of dioxan-based scintillator [containing, per litre, 0.2 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 4 g of 2,5-diphenyloxazole, 60 g of naphthalene and 200 ml of ethanol] was added to each of the vials, and the radioactivity was measured with a Nuclear-Chicago mark 2 counter.

Results and Discussion

The incubation of factor F_1 with 1 mM mixed anhydride of ATP (or 1, N^6 -etheno-ATP) and mesitylenecarboxylic acid for 1 h leads to the 90–95% inhibition of the ATPase activity of the enzyme (Kozlov *et al.*, 1977, 1979). This inhibition is accompanied by covalent binding of 1 mol of the inhibitor/mol of the enzyme. ATP protects factor

F_1 from the binding of the mixed anhydride of 1, N^6 -etheno-ATP and mesitylenecarboxylic acid (Kozlov *et al.*, 1979). The bond between the residue of the inhibitor and factor F_1 remained stable during treatment of the modified enzyme with cold 5% (w/v) trichloroacetic acid or during dialysis for several days against 8 M-urea (Kozlov *et al.*, 1979).

Similar results were obtained in the presence study. Incubation of factor F_1 with the mixed anhydride of [^3H]ATP and mesitylenecarboxylic acid leads to the covalent binding of 0.9 mol of the inhibitor to 1 mol of factor F_1 . Electrophoresis of modified factor F_1 in polyacrylamide gel in the presence of sodium dodecyl sulphate (Fig. 1) showed that the mixed anhydride of [^3H]ATP and mesitylenecarboxylic acid binds to the β -subunit of the enzyme.

It has already been shown that the mixed anhydrides of mesitylenecarboxylic acid and ADP or AMP do not inhibit isolated factor F_1 (Kozlov *et al.*, 1977, 1979). On the other hand, the mixed anhydride of adenosine 5'-[$\beta\gamma$ -methylene]triphosphate and mesitylenecarboxylic acid inhibits factor F_1 as well as does the mixed anhydride of ATP and mesitylenecarboxylic acid (Kozlov *et al.*, 1977). These results indicate that the reaction of factor F_1 with the mixed anhydride of ATP and mesitylenecarboxylic acid is accompanied by the covalent binding of the ATP residue of the inhibitor to the enzyme molecule.

Thus the mixed anhydride of ATP and mesitylenecarboxylic acid modifies some nucleophilic group of

Fig. 1. Electrophoresis of factor F_1 , modified by the mixed anhydride of [^3H]ATP and mesitylenecarboxylic acid, in polyacrylamide gel in the presence of sodium dodecyl sulphate

Factor F_1 treated with 1 mM mixed anhydride of [^3H]ATP and mesitylenecarboxylic acid in 20 mM-Mops/KOH buffer, pH 7.5, containing 0.25 M-sucrose and 2 mM-EDTA for 60 min was separated from excess of the labelled ATP analogue on a Sephadex G-25 column. A 50 μg sample of modified factor F_1 was applied to the gel. The continuous black line represents the scanning at 630 nm of gel stained with Amido Black. The hatched rectangles show the radioactivity of the gel slices.

factor F_1 located at or near the site of binding of the γ -phosphate group of the inhibitor molecule. The results obtained in this work indicate that the binding site for the γ -phosphate residue of ATP is localized on the β -subunit of factor F_1 .

We thank Professor V. P. Skulachev for discussion of the results and Mrs. G. A. Kozlov for translation of the paper.

References

- Budker, V. G., Kozlov, I. A., Kurbatov, V. A. & Milgrom, Ya. M. (1977) *FEBS Lett.* **83**, 11–14
- Catterall, W. A. & Pedersen, P. L. (1971) *J. Biol. Chem.* **246**, 4987–4994
- Esch, F. S. & Allison, W. S. (1978) *J. Biol. Chem.* **253**, 6100–6106
- Ferguson, S. J., Lloyd, W. J., Lyons, M. H. & Radda, G. K. (1975) *Eur. J. Biochem.* **54**, 117–126
- Horstman, L. L. & Racker, E. (1970) *J. Biol. Chem.* **245**, 1336–1344
- Kemp, A., Jr. (1978) in *Bioenergetics at Mitochondrial and Cellular Levels* (Wojtczak, L., Lenartowicz, E. & Zborowski, J., eds.), pp. 103–131, Institute of Experimental Biology, Warsaw
- Knowles, A. F. & Penefsky, H. S. (1972) *J. Biol. Chem.* **247**, 6624–6630
- Kozlov, I. A. & Mikelsaar, H. N. (1974) *FEBS Lett.* **43**, 212–214
- Kozlov, I. A. & Skulachev, V. P. (1977) *Biochim. Biophys. Acta* **463**, 29–89
- Kozlov, I. A., Shalamberidze, M. V., Novikova, I. Yu., Sokolova, N. I. & Shabarova, Z. A. (1977) *Biokhimiya* **42**, 1704–1710
- Kozlov, I. A., Shalamberidze, M. V., Novikova, I. Yu., Sokolova, N. I. & Shabarova, Z. A. (1979) *Biochem. J.* **179**, 339–343
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lunardi, J., Lauquin, G. J. M. & Vignais, P. V. (1977) *FEBS Lett.* **80**, 317–323
- Pedersen, P. L. (1975) *J. Bioenerg.* **6**, 243–275
- Racker, E. (1965) *Mechanisms in Bioenergetics*, Academic Press, New York and London
- Senior, A. E. (1973) *Biochim. Biophys. Acta* **301**, 249–277
- Slater, E. C. (1978) *Abstr. FEBS Meet. 12th Abstr.* 0526
- Tretyakova, S. S., Drutsa, V. L., Lebedev, A. V., Sokolova, N. I. & Shabarova, Z. A. (1978) *Bioorg. Khim.* **4**, 911–916
- Wagenvoort, R. J., Van der Kraan, I. & Kemp, A. (1977) *Biochim. Biophys. Acta* **460**, 17–24
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412