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STRUCTURAL REARRANGEMENTS IN SOLUBLE MITOCHONDRIAL ATPase

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Summary

Treatment of isolated factor F_1 by 1% dimethylsuberimidate in the presence of 50 mM $(NH_4)_2SO_4$ leads to the formation of four different types of cross-linked dimers of the subunits, on average one dimer per molecule of the enzyme. This treatment results in 60—70% inactivation of factor F_1. Factor F_1 treated with dimethylsuberimidate does not show a change in the sedimentation coefficient and is not inactivated in the cold; it is not inactivated in the presence of Mg^{2+} either, nor is it activated by anions. Incubation of the cross-linked factor F_1 with ADP does not lead to inactivation, although the ability to tightly bind ADP is retained. The total quantity of tightly bound ADP reaches 5 mol per mol of the cross-linked factor F_1.

Cross-linking of factor F_1 also prevents the slow inactivation of the enzyme coupled with the hydrolysis of Mg-ATP and Mg-GTP. The dependence of the inactivation rate constant on the concentration of Mg-ATP and Mg-GTP at substrate concentrations of 0.05—2 mM is characterized by the same values of $K_{m,app}$ as those of the ATPase and GTPase activities of factor F_1. The probability of the inactivation of factor F_1 per turnover remains constant for all the concentrations of the substrates studied and is $2 \cdot 10^{-6}$ per turnover for the ATPase reaction and $2 \cdot 10^{-5}$ per turnover for the GTPase reaction. Moderate hydrostatic pressure (up to 150 atmospheres) greatly accelerates ATP-induced inactivation of factor F_1. The activation volume (ΔV^*) of the inactivation process is equal to $5.1 \cdot 10^{-4}$ cm^3/g, which is evidence of considerable changes in the extent of protein hydration during inactivation. Inactivation of the enzyme under pressure is accompanied by dissociation into subunits.

Dimethyladipimidate, which does not cause intersubunit cross-linking in the

molecule of factor F_1, does not alter the properties of the native enzyme.

It is suggested that the formation of one intersubunit cross-link in the molecule of factor F_1 by dimethylsuberimidate affects the ability of the enzyme to undergo co-operative rearrangements of the quaternary structure under the influence of Mg^{2+}, ADP, ATP, anions, and low temperature.

The rate constants of ATP binding to the active site of factor F_1 (k_{+1}) = $2 \cdot 10^8$ $M^{-1} \cdot min^{-1}$), of ATP release from the active site ($k_{-1} = 2 \cdot 10^{-2}$ min^{-1}), and of ADP and P_i release from the active site ($k_2 = 5 \cdot 10^3$ min^{-1}) have been determined. The results obtained confirm the correctness of Boyer's idea, according to which ATP is formed in the active site of mitochondrial ATPase without any external source of energy. Energy is used at the stage of the release of synthesized ATP from the active site of ATPase in the solution.

Introduction

Factor F_1 (soluble mitochondrial ATPase) is the component of the ATPase complex of mitochondria that catalyses the synthesis of ATP in oxidative phosphorylation. It has a molecular weight of about 370 000 and consists of five types of subunits: α(55 000), β(51 000), γ(33 000), δ(17 000), and ϵ(11 000) (for reviews see Refs. 1 and 2). It has been suggested that the subunit composition of the enzyme molecule is described by the formula $\alpha_3\beta_3\gamma\delta\epsilon$ [3,4].

Isolated factor F_1 takes part in at least two processes connected with the mechanism of oxidative phosphorylation: the enzyme hydrolases Mg-ATP at a high rate; and it binds ADP, the substrate of oxidative phosphorylation, with a high affinity and a high specificity. Both of these processes are accompanied by conformational changes in the enzyme molecule (for reviews see Refs. 2, 5 and 6).

To determine the part played by the polysubunit structure of factor F_1 in these conformational changes, we used the bifunctional cross-linking reagent dimethylsuberimidate, which forms intersubunit cross-links in the enzyme.

Bifunctional cross-linking reagents have been used to study the structures of soluble ATPase from bovine heart [7,8] and yeast [9] mitochondria, from chloroplasts [10], *Escherichia coli* [11] and also of the oligomycin-sensitive ATPase from yeast mitochondria [9].

In the present paper the conformational changes in isolated factor F_1 induced by Mg^{2+} [12,13], certain anions [13,14], ADP [15] or cold [16] were studied. The results obtained demonstrate that these changes are coupled with major co-operative rearrangements in the quaternary structure of the enzyme.

A study is made of the slow conformational changes induced by Mg-ATP which are accompanied by the inactivation of the enzyme. These changes are shown to affect the quaternary structure of ATPase and occur with a frequency of one per $2 \cdot 10^6$ turnovers of factor F_1 during ATP hydrolysis. Slow inactivation of factor F_1 in the course of the ATPase reaction was also observed by Beechey and co-workers [17].

Indications have also been obtained that the binding of the substrate in the non-catalytic site of factor F_1 leads to an increase in the rate of the ATPase reaction and to a decrease in the affinity of the active site to Mg-ATP. The

values of the rate constants of the individual steps in the ATPase reaction have been determined. Some of the preliminary results of this work have been published [18].

Materials and Methods

Factor F_1 was isolated according to Horstman and Racker [19] or Knowles and Penefsky [4]. Protein was determined according to the method of Lowry et al. [20]. To determine the content of tightly bound nucleotides, the preparation of the enzyme, after chromatography on a column of Sephadex G-50, was treated with 3% trichloroacetic acid; the protein pellet was removed by centrifugation, and the supernatant neutralized with 5 N KOH. The ATP and ADP content in the supernatant was determined in standard coupled enzyme systems [5]. To desalinate the preparation of factor F_1, the protein was placed in a column of Sephadex G-25, equilibrated with a solution containing 50 mM Hepes and 0.25 M sucrose, pH 8.5. The ammonium sulphate content was determined with the Nessler reagent [21].

Electrophoresis was conducted in 10% polyacrylamide gel with sodium dodecyl sulphate according to Weber and Osborn [22]; the gels were stained with amido black and scanned with a Gilford spectrophotometer at a wavelength of 630 nm. The area of the peaks on the densitograms was proportional to the amount of factor F_1 placed in the tube (from 20 to 100 μg protein).

To determine the molecular weights of the oligomers of subunits of factor F_1 that were cross-linked with dimethylsuberimidate, the gels were calibrated with the following proteins; phosphorylase *a* from rabbit muscle (molecular weight of the monomer 92 000). Human IgM macroglobulin, heavy chain (72 000), bovine serum albumin (65 000), G-actin (46 000), light chain of IgM macroglobulin (23 000), and cytochrome *c* (12 500). The molecular weights of the subunits of factor F_1 determined according to the calibration curve were 59 000 (α), 51 000 (β), and 29 000 (γ).

The electrophoresis of factor F_1 in 7.5% polyacrylamide gel was carried out at pH 8.3 in the absence of detergents. Pre-electrophoresis was performed preliminarily for 3—4 h. The gels were stained with amino black or examined for ATPase activity. For this purpose, the gels were rinsed with water and incubated 15—20 min in a mixture containing 2 mM ATP, 2 mM $MgSO_4$ and 10 mM Tris-HCl, pH 8.3; then they were washed with water and stained to determine inorganic phosphate content according to the method of Sugino and Miyoshi [23].

The experiments on the sedimentation of the enzyme were conducted in a Spinco Model E analytical ultracentrifuge with a scanning optical system.

The ATPase activity was usually determined pH-metrically, as described earlier [24], or in an ATP-regenerating system. Details are given in the figure captions. The rate of the ATPase reaction was measured for 1—2 min, during which time the ATPase activity of factor F_1 did not change. The absorbance during NADH oxidation was measured at a wavelength of 340 nm with a Hitachi-124 or Specord UV/Vis spectrophotometer. The concentration of the Mg-ATP complex in these experiments was calculated using a dissociation constant of $5 \cdot 10^{-5}$ M [25].

The effect of hydrostatic pressure on ATPase was studied in a cuvette, prepared from a standard cell of a Spinco-E ultracentrifuge and specially fortified. The volume of the cuvette was about 2 ml, the length of the optical path was 1.2 cm. Pressure was created with compressed nitrogen and was measured with a pressure gauge (marked in 5 atmospheres). The ATPase activity under pressure was measured in an ATP-generating system. In the preliminary experiments it was shown that a pressure of up to 150 atmospheres does not have any significant effect on the activity of pyruvate kinase and lactate dehydrogenase.

Dimethyladipimidate (Polysciences Inc.), dimethylsuberimidate (Pierce), and 5′-adenylyl imidodiphosphate (Ado*PP*[NH]*P*) (Sigma) were used in this work. The mixed anhydride of ATP and mesitylene carboxylic acid was kindly supplied by Dr. N.I. Sokolova and Dr. Z.A. Shabarova. The remaining reagents from Reanal and Reachim were of maximal purity.

Results

The cross-linking of factor F_1 with dimethylsuberimidate

In this work a study was made of the reaction of factor F_1 with bifunctional cross-linking reagents of different lengths: dimethyladipimidate with a cross-link 8 Å in length, and dimethylsuberimidate with a cross-link 11 Å in length.

Dimethyladipimidate in a concentration of up to 1% did not inhibit ATPase. Factor F_1 treated with 1% dimethyladipimidate for 1 h was inactivated in the cold in the presence of 0.5 M KCl at the same rate as the untreated enzyme. Electrophoresis in the presence of sodium dodecyl sulphate showed that the modification of factor F_1 by dimethyladipimidate did not lead to the formation of intersubunit cross-linking (Fig. 1, scan 1). Treatment with dimethylsuberimidate at a concentration of 0.5% or more led to a rapid and complete inactivation of the ATPase activity (Fig. 2, curve 1). ATP at a concentration of 10 mM, and Mg-ADP at a concentration of 2 mM, did not protect the ATPase from inactivation. This treatment led to the formation of covalently cross-linked complexes with molecular weights of 200 000 and higher (not shown). Those subunits that did not undergo cross-linking were less than 30% of the total protein. To decrease the probability of cross-linking, ammonium salts reacted with imidates were added.

Treatment with dimethylsuberimidate in the presence of 50 mM $(NH_4)_2SO_4$ or 100 mM NH_4Cl resulted in only 60—70% inactivation (Fig. 2, curve 2). At this concentration dimethylsuberimidate in the absence of ammonium salts or in the presence of KCl or K_2SO_4 instead of ammonium salts completely inactivated the ATPase in 15 min (not shown).

1% dimethyladipimidate did not cause inhibition of the ATPase in the presence of $(NH_4)_2SO_4$ (Fig. 2, curve 3). The treatment of factor F_1 with 1% dimethyladipimidate for 1 h prevented cross-linking and the inactivation of the enzyme by 1% dimethylsuberimidate (Fig. 2, curve 4). Electrophoresis with sodium dodecyl sulphate of factor F_1 modified by 1% dimethylsuberimidate in the presence of 50 mM $(NH_4)_2SO_4$ revealed five new protein bands, with molecular weights of approx. 140 000, 110 000, 105 000, 89 000, 82 000 (Fig. 1). The molecular weights of the last four bands can only correspond to dimers of the subunits of factor F_1. The fifth band consists of less than 3% of all the pro-

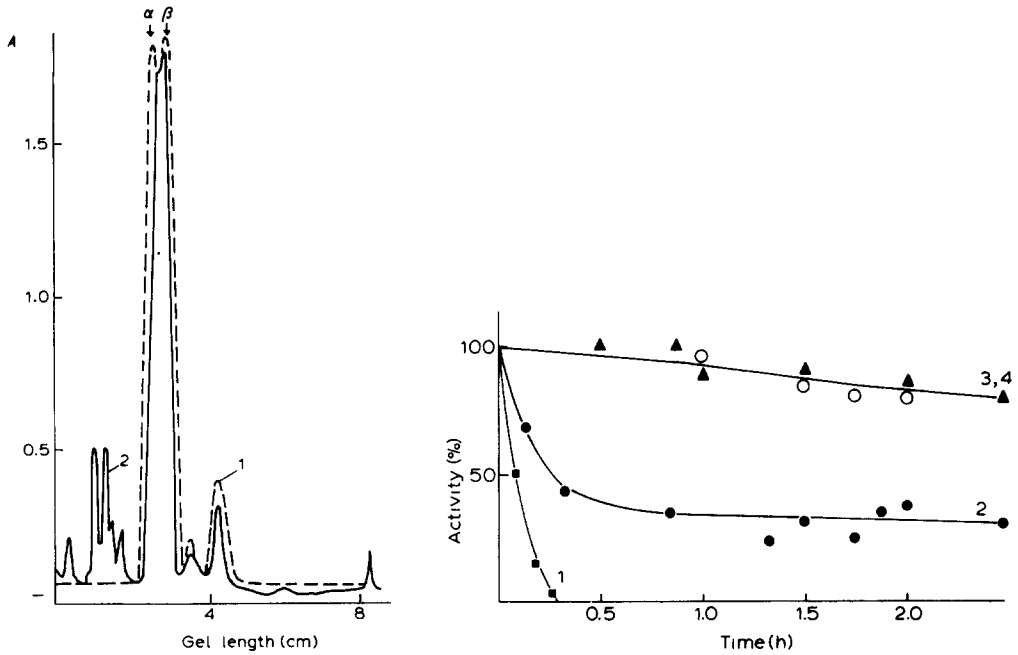


Fig. 1. Electrophoresis in the presence of sodium dodecyl sulphate of factor F_1 treated with cross-linking reagents. 1, - - - - -, factor F_1 treated with dimethyladipimidate (a similar picture is observed for native factor F_1). 2, ———, factor F_1 treated with dimethylsuberimidate. The treatment of factor F_1 (1 mg/ml) with 1% dimethyladipimidate or 1% dimethylsuberimidate was carried out in 100 mM Hepes-buffer, pH 8.5 in the presence of 50 mM $(NH_4)_2SO_4$ for 90 min. Approx. 70 μg protein was added to the gel tube. Electrophoresis was carried out for 18 h at a current of 3 mA per tube.

Fig. 2. Inhibition of factor F_1 by dimethylsuberimidate. Curve 1, 0.5% dimethylsuberimidate. Curve 2, 1% dimethylsuberimidate in the presence of 50 mM $(NH_4)_2SO_4$. Curve 3 (▲), 1% dimethyladipimidate in the presence of 50 mM $(NH_4)_2SO_4$. Curve 4 (○), 1% dimethylsuberimidate was added to factor F_1 preincubated for 1 h with 1% dimethyladipimidate in the presence of 50 mM $(NH_4)_2SO_4$. The treatment of factor F_1 with diimidates was carried out in 100 mM Hepes buffer, pH 8.5 at a protein concentration of 1—1.5 mg/ml. After the intervals of time indicated small aliquots were taken from mixture to determine ATPase activity. The initial ATPase activity of factor F_1 was 50 μmol · min^{-1} · mg^{-1} protein at 25°C. The content of the medium to measure ATPase activity is indicated in the explanation to Table I.

tein and corresponds to higher molecular weight product(s) of cross-linking. According to the data of Satre et al. [8], the dimers of F_1 subunits with molecular weights of 100 000—115 000 that are formed as a result of cross-linking by dimethylsuberimidate have a composition of αα and αβ. It may be suggested that the dimers with molecular weights of 80 000—90 000 consist of large and minor subunits of factor F_1.

The cross-linked dimers of the subunits consist of about 20% of the overall protein mass; consequently, the treatment of factor F_1 with dimethylsuberimidate in the presence of 50 mM $(NH_4)_2SO_4$ leads to the formation on average of one dimer of the subunits per molecule of enzyme.

The stabilising effect of dimethylsuberimidate on factor F_1

Factor F_1 treated with 1% dimethylsuberimidate in the presence of 50 mM $(NH_4)_2SO_4$, and possessing 30—40% of the initial activity, becamse resistant to

low temperatures while the native enzyme, and also that treated with dimethyladipimidate or treated successively with dimethyladipimidate and dimethylsuberimidate, was rapidly inactivated at 0°C in the presence of 0.5 M KCl (Table I). Sedimentation experiments show that the inactivation of the native factor F_1 in the cold is accompanied by the disappearance of a 12-S component and the formation of a 3-S component, which demonstrates the dissociation of the enzyme into subunits [16]. The modification by 1% dimethylsuberimidate in the presence of 50 mM $(NH_4)_2SO_4$ caused factor F_1 to remain resistant to cold for 1.5—2 h and not dissociate into subunits. Complete inactivation only occurs when the concentrations of KCl is raised to 2 M and the incubation time at 0°C is increased. This inactivation of the cross-linked factor F_1 is accompanied by dissociation.

The preparation of factor F_1 treated with dimethylsuberimidate and incubated in cold (0°C, 0.5 M KCl, 1 h) was subjected to electrophoresis at pH 8.3 with the subsequent determination of the ATPase activity of the protein bands in the gel. The electrophoretic mobility of the zone with ATPase activity corresponded to the mobility of the undissociated factor F_1. The results obtained demonstrated that only whole molecules of factor F_1 possess ATPase activity. A similar conclusion can be drawn from the results of the study of factor F_1 by the method of sedimentation of the active enzyme which showed that, in the preparation of factor F_1 modified by dimethylsuberimidate and subjected to incubation for 1 h at 0°C in the presence of 0.5 M KCl, only the component with a sedimentation coefficient of 12.4-S possessed ATPase activity [26].

Treatment of factor F_1 with dimethylsuberimidate in the presence of $(NH_4)_2SO_4$ made the enzyme resistant to certain effectors of the ATPase activity. Thus, treatment with dimethylsuberimidate abolished the activating effect of certain anions and prevented the inactivation of the enzyme by Mg^{2+} in pre-

TABLE I

THE STABILIZING EFFECT OF DIMETHYLSUBERIMIDATE ON FACTOR F_1

The initial ATPase activity of the factor F_1 preparations was: without treatment, 45 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein; after dimethylsuberimidate treatment 17 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein, and after dimethyladipimidate, or dimethylsuberimidate following dimethyl adipimidate treatment, 40 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein. The ATPase activity was determined pH-metrically [24]. The reaction mixture medium contained factor F_1 (0.5—1 $\mu g/ml$), 2 mM ATP, 2 mM $MgSO_4$, 3 mM Tris-HCl, pH 8.3 in a final volume of 8 ml. The conditions of factor F_1 treatment with diimidates are given in the caption to Fig. 1. For cold inactivation factor F_1 (1—1.5 mg/ml) was incubated at 0°C in 100 mM Hepes buffer, pH 8.3 in the presence of 0.5 M KCl. Preincubation with 1 mM $MgSO_4$ was carried out in the same medium without KCl. Activation by anions was observed with 10 mM $KHCO_3$ or 5 mM K_2SO_3 in the reaction mixture.

Treatment	ATPase activity (%)		
	After 0.5 h in cold	+ HCO_3^- or SO_3^{2-}	After 1 h incubation with Mg^{2+}
None	5	300	30
Dimethyl suberimidate	90	100	95
Dimethyladipimidate (or dimethylsuberimidate after dimethyladipimidate)	15	300	30

incubation (Table I). Factor F_1 modified by dimethyladipimidate or successively by dimethyladipimidate and dimethylsuberimidate was effected by anions and Mg^{2+}, just like the control preparation of the enzyme (Table I).

Nucleotide binding by factor F_1

Factor F_1 was inactivated by 60—70% in 1—2 min in the presence of 0.4 mM ADP; this effect was reversible, when 2 mM ATP was added to the incubation medium the activity was almost completely restored. Factor F_1 treated with dimethylsuberimidate was not inactivated in the presence of ADP and the treatment with dimethyladipimidate did not prevent inactivation (Table II).

The incubation of native factor F_1 initially containing about 2 mol of tightly bound ADP per mol of enzyme with ADP in conditions of inhibition of its ATPase activity led to the additional tight binding of approx. 1 mol of ADP per mol of factor F_1 (Table II). Factor F_1 preincubated in the presence of ADP and inorganic phosphate bound 3—4 mol of ADP additionally without inactivation (Table II). Treatment of factor F_1 with dimethylsuberimidate resulted in a similar effect. The cross-linked enzyme bound two to three additional mol of ADP per mol of factor F_1, but this binding did not lead to inactivation (Table II).

Inactivation of factor F_1 in the course of the ATPase reaction

Fig. 3 shows that the Mg-ATP hydrolysis of factor F_1 in an ATP-regenerating system is accompanied by inactivation of the enzyme. This inhibition cannot be explained by the accumulation of any rapidly acting inhibitors of ATPase, since when a new aliquot of the enzyme is added to the reaction mixture ATP hydrolysis occurs at a maximal rate (Fig. 3, curve 1). In the same reaction mix-

TABLE II

THE TIGHTLY BOUND ADP CONTENT IN FACTOR F_1

The incubation of factor F_1 (5—6 mg/ml) with ADP was carried out in a medium containing 0.1 M Hepes, pH 6.7 and 0.25 M sucrose for 15 min at room temperature. After separation of the free ADP by gel filtration on a Sephadex G-50, the content of tightly bound ADP in factor F_1 was determined as described in Methods. Five different preparations of factor F_1 were used, and the results were given as mean values ± S.E. The content of tightly bound ATP in the preparations of factor F_1 was no more than 0.5 mol of ATP/mol of the enzyme. The ATPase activity was measured before gel filtration in an ATP-regenerating system containing 50 mM Tris-HCl, pH 7.5, 7.5 mM KCl, 2 mM ATP, 2.5 mM $MgSO_4$, 0.4 mM NADH, 0.5 mM phospho*enol*pyruvate, 3 U/ml pyruvate kinase, 2 U/ml lactate dehydrogenase. The maximum inhibition was achieved by the fifth minute of incubation and was fully reversed by the addition of 2 mM of ATP to the preincubation mixture (not shown). The treatment of factor F_1 with dimethylsuberimidate was carried out as indicated in the caption to Fig. 1.

Preparation	Incubation conditions	Mol tightly bound ADP per mol factor F_1	Extent of inhibition of factor F_1 as a result of incubation with ADP
Factor F_1	without additions	2.0 ± 0.5	
Factor F_1	0.4 mM ADP	3.2 ± 0.2	40—50%
Factor F_1	0.4 mM ADP and 20 mM KH_2PO_4	5.5 ± 0.3	10—20%
Factor F_1 treated with dimethylsuberimidate	0.4 mM ADP	4.9 ± 0.4	no inhibition

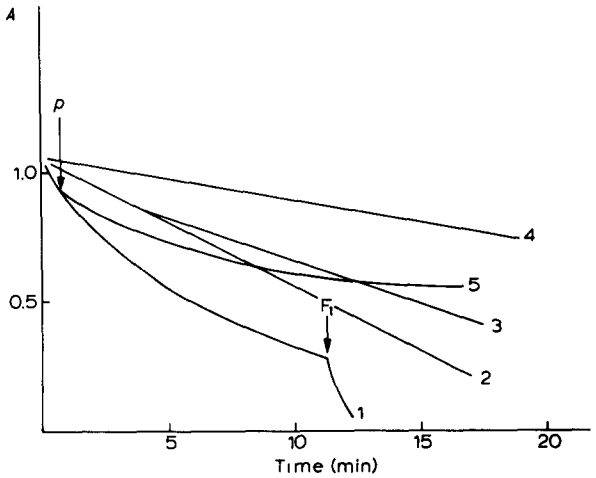


Fig. 3. Mg-ATP-induced inactivation of factor F_1. For the composition of the medium to measure ATPase activity see caption to Table II, and for the conditions regarding the treatment of factor F_1 with dimethysuberimidate see caption to Fig. 1. Measurements were made on Specord UV-VIS (GDR) spectrophotometer. The volume of the sample was 2 ml, and the concentration of factor F_1 in the sample 1 μg/ml. Curve 1, control preparation of factor F_1. The arrow indicates the moment when a new addition of factor F_1 (2 μg protein) was made. The concentration of Mg-ATP was 2 mM. Curve 2, factor F_1 treated wtih 1% dimethylsuberimidate in the presence of 50 mM $(NH_4)_2SO_4$ and then preincubated for 1 h at 0° C in the presence of 0.5 M KCl. The concentration of Mg-ATP was 2 mM. Curve 3, control preparation of factor F_1. The concentration of Mg-ATP was 0.16 mM. Curve 4, control preparation of factor F_1. The concentration of Mg-ATP was 0.025 mM. Curve 5, conditions as for Curve 1, but at the moment indicated by the arrow a pressure of 150 atm was applied.

ture, but in the absence of the substrate or in the presence of ATP but without Mg^{2+}, inactivation of factor F_1 does not occur. The inactivation of factor F_1 cannot be due to the free Mg^{2+} present in the reaction mixture, since its concentration is not higher than 0.3 mM, which is considerably lower than the inhibitory concentration [12]. The stationary concentration of ADP in the standard conditions of a regenerating system did not exceed 0.6 μM. The lowering of its concentration to $1.5 \cdot 10^{-3}$ μM by increasing the amount of pyruvate kinase did not change the time course of inactivation. Therefore, the inactivation of factor F_1 in our experiments is not induced by ADP.

At saturating concentrations of the substrate (1—2 mM), beginning with the tenth second and up to 20—25 min of incubation, the rate of ATP hydrolysis decreases exponentially, $V_t = V_0 \cdot (\exp -kt)$ (where k is the inactivation rate constant). At Mg-ATP concentrations lower than 0.025 mM inactivation is hardly observed at all for 30—40 min (Fig. 3, curve 4). At substrate concentrations of 0.05—0.15 mM inactivation is only observed during the first 10—15 min after the beginning of the reaction; then the inactivation rate drops, and from 15—20 min onwards the hydrolysis takes place at an almost constant rate (Fig. 3, curve 3). Such an inactivation time course indicates that at these substrate concentrations after 10—15 min of incubation an equilibrium is established between the active and inactive forms of the enzyme. The position of this equilibrium and the time that is needed to reach this equilibrium depend on the substrate concentration. During the first 10 min inactivation is approximately exponential. In these conditions when the system is far from equilib-

rium the initial inactivation rate constant (k) could be determined. The dependence of $1/k$ on 1/[S] describes a complex curve with two linear sections in the substrate concentration intervals of 2—0.05 mM and 0.05—0.02 mM. The values of the apparent Michaelis constants ($K_{m,app}$) of the inactivation process for these intervals are 0.25 mM and 0.026 mM (Fig. 4, curve 1). The dependence of $1/k$ on 1/[S] for Mg-GTP-induced inactivation of factor F_1 is linear for all the substrate concentration studied, with $K_{m,app}$ equal to 0.22 mM (Fig. 4, curve 2).

For the inactivation process the continual presence of the substrate in the incubation medium is needed. Short incubation (from 30 s to 2 min) of factor F_1 in the presence of saturating concentrations of Mg-ATP (2 mM), with the subsequent addition of an excess of EDTA (4 mM) to the incubation medium, did not lead to an inactivation of the enzyme; when the reaction was started again after 15—20 min by adding $MgSO_4$ (4 mM), the ATP hydrolysis rate was similar to that observed before the EDTA addition.

The reversible inhibitor of ATPase, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazol, prevents substrate-induced inactivation. Factor F_1 preincubated with 100 μM of this inhibitor for 1 h at pH 8.0 (97—98% inhibition) was placed in a reaction medium containing 2 mM of Mg-ATP and incubated for 15—20 min. The subsequent addition of 1 mM dithiothreitol, which reverses the effect of the inhibitor, resulted in the restoration of the initial ATPase activity. According to the data of Ferguson et al. [27] factor F_1 modified by 7-chloro-4-nitrobenzo-2-oxa-1,3-diazol retains its ability to bind Mg-ATP. Thus, the substrate induced inactivation of factor F_1 is coupled with one of the stages of the ATPase reac-

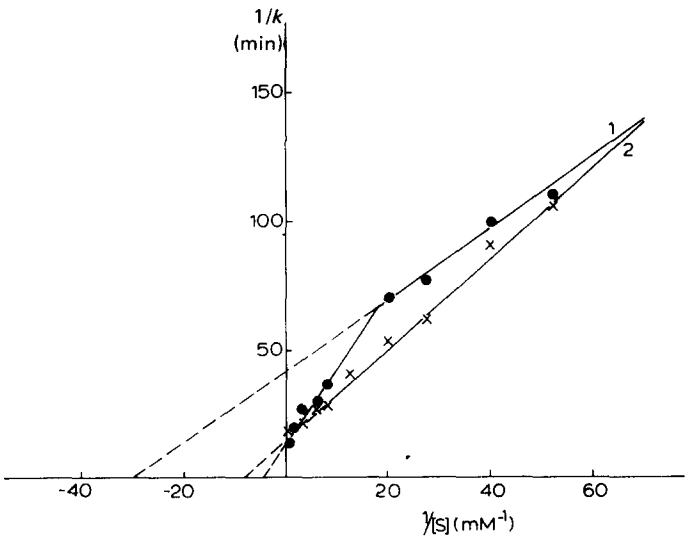


Fig. 4. The dependence of the inverse value of the rate constant of factor F_1 inactivation on the inverse substrate concentration. The values of k of ATP- or GTP-induced inhibition obtained from the semilogarithmic plots of V_t on t where v_t is the reaction rate at time t was determined by graphic differentiation of the reaction curves. Measurement conditions as described in caption to Fig. 3. The concentration of the Mg-ATP complex was calculated using a dissociation constant of $5 \cdot 10^{-5}$ M [25]. Curve 1, ATP-induced inhibition; Curve 2, GTP-induced inhibition.

tion following the formation of the enzyme-substrate complex.

Preincubation of factor F_1 with ADP, which causes the additional tight binding of 1 mol of ADP per mol of factor F_1 (Table II) or with Mg^{2+} did not prevent substrate-induced inactivation of the enzyme. Inactivation of factor F_1 during ATP hydrolysis can also be observed in the presence of activating anions. Glycerine and poly(ethylene glycol) slow down the rate of inactivation.

The treatment of factor F_1 with dimethylsuberimidate under conditions leading to the formation of one intersubunit bond per molecule of factor F_1 prevents the Mg-ATP-induced inactivation of the enzyme (Fig. 3, curve 2). The effect is not connected with the partial inhibition of the ATPase reaction caused by the dimethylsuberimidate treatment. In actual fact, when the substrate concentration was lowered to 0.16 mM, the ATPase reaction rate dropped to the level of activity of the enzyme inhibited by dimethylsuberimidate; however, Mg-ATP-induced inactivation of factor F_1 continued (Fig. 3, curves 2 and 3).

The effect of dimethylsuberimidate is not due simply to a modification of the amino groups, since dimethyladipimidate had no effect on the inactivation process (not shown).

It appeared that the Mg-ATP-induced inactivation of factor F_1 was considerably accelerated if the pressure in the measuring cells was raised to 100—150 atm (Fig. 3, curve 5). The subsequent relief of pressure did not cause any reactivation. The dependence of the rate constant of inactivation on pressure is described by the equation:

$$k = k_0 \cdot (\exp(-\Delta V^* \cdot P/RT))$$

where k_0 is the rate constant of inactivation at atmospheric pressure. ΔV^*, the activation volume of the inactivation reaction; T, the absolute temperature; P, the pressure; R, the universal gas constant.

The plot $\ln k/k_0$ vs. P (Fig. 5) makes it possible to determine the value of ΔV^*, which is equal to $5.1 \cdot 10^{-4}$ cm^3/g (assuming a molecular weight of factor F_1 of 360 000 [4]).

The results given in Fig. 5 were obtained with a concentration of Mg-ATP equal to 2 mM. AT an Mg-ATP concentration of 0.02 mM, i.e. when the ATP hydrolysis at atmospheric pressure is not accompanied by the inactivation of the enzyme for 30—40 min (Fig. 3, curve 4), an increase in pressure to 150 atm does not cause any noticeable inactivation either. At Mg-ATP concentrations of 0.05—0.2 mM, which cause partial inactivation of the enzyme with the subsequent establishment of equilibrium between the active and inactive forms, the increase in pressure leads to almost complete inactivation of the enzyme.

As the experiments showed, a pressure of up to 150 atm only causes slight (up to 30%) irreversible inactivation of the enzyme in the absence of the substrate or in the presence of ATP, but without Mg^{2+}. Nor did this pressure lead to inactivation of the reacting factor F_1 modified by dimethylsuberimidate. However, the Mg-ATP-induced inactivation of the protein modified by dimethyladipimidate was accelerated by this pressure just as was that of the native protein.

The changes occurring in the reacting factor F_1 under pressure were also studied by the method of electrophoresis in polyacrylamide gel. The data ob-

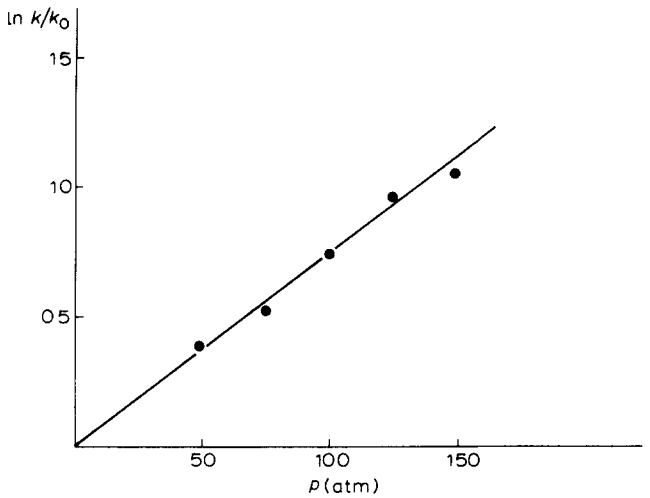


Fig. 5. The dependence of the inactivation rate constant on pressure. k, the inactivation rate constant at a given pressure. k_0, the inactivation rate constant at atmospheric pressure. The values of k for the different pressures obtained as described in the caption to Fig. 4. For the composition of the measurement medium, see the caption to Table II. Measurements were made on a Specord UV/VIS spectrophotometer in a special cuvette (see Methods).

tained in these experiments are given in Fig. 6. Under the conditions of the experiment there was only 20% Mg-ATP-induced inactivation of factor F_1 at atmospheric pressure, and there was no noticeable dissociation of the enzyme into subunits (Fig. 6, scans 1 and 2). At a pressure of 150 atm but with otherwise identical conditions there was almost complete dissociation of factor F_1 (scan 4). This pressure in the absence of the substrate causes only partial dissociation of the enzyme into subunits (scan 3).

Dependence of ATPase activity on substrate concentration

In the case of native factor F_1, the dependence of the ATPase activity on the concentration of Mg-ATP in the interval of 0.01—2 mM did not obey Michaelis-Menten kinetics (Fig. 7, curve 1). In the substrate concentration interval of 0.01—0.1 mM, the hydrolysis of ATP occurred with a $K_{m,app}$ equal to 0.03 mM and a V equal to 14 μmol/min per mg protein. The hydrolysis at higher concentrations of the substrate occurred with a $K_{m,app}$ equal to 0.3 mM and V equal to 34 μmol/min per mg protein. The preincubation of factor F_1 with 0.4 mM ADP for 15 min, which is accompanied by 60% inactivation of the enzyme and additional binding of 1 mol of ADP per mol of the enzyme (see Table II), did not alter the character of the dependence of ATPase activity on the substrate concentration and $K_{m,app}$ values.

The character of the ATPase activity as a function of substrate concentration and the values of $K_{m,app}$ did not change as a result of the partial (60% or 80%) inhibition of factor F_1 by the mixed anhydride of ATP and mesitylene carboxylic acid, which modifies the active site of the enzyme [28,29].

Treatment of factor F_1 with 1% dimethylsuberimidate in the presence of $(NH_4)_2SO_4$ led to a decrease in V of the ATPase reaction. The dependence of

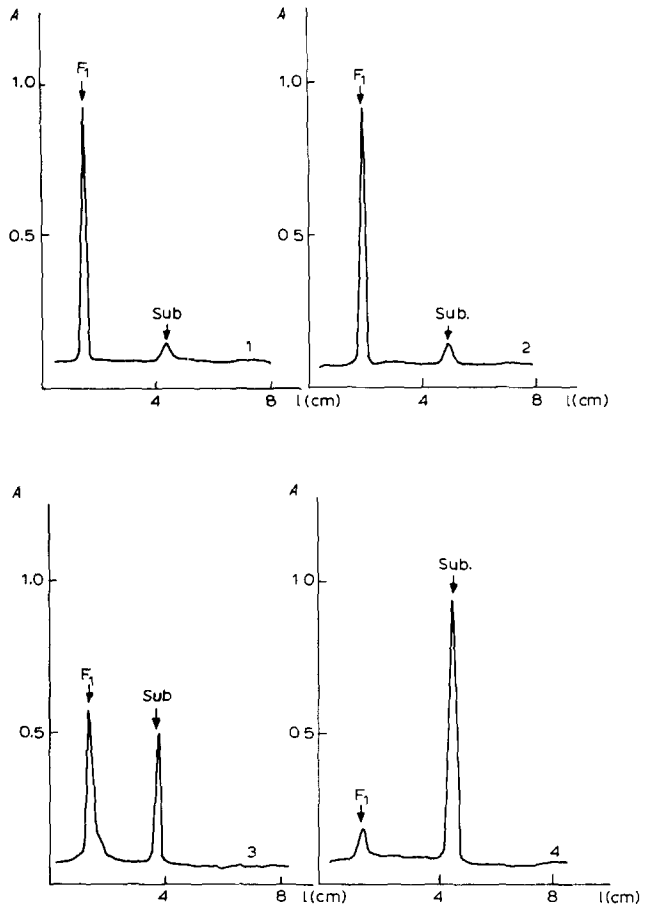


Fig. 6. Electrophoresis in polyacrylamide gel of factor F_1 inactivated at various pressures. Staining was carried out with amido black and scanning at 630 nm. 20—30 μg of protein was placed in the tube. 1, factor F_1 was incubated at a concentration of 200 μg/ml for 30 min in a medium containing 50 mM Tris-HCl and 0.2 mM EDTA, pH 7.5. 2, factor F_1 was incubated at a concentration of 200 μg/ml for 30 min in a medium containing 50 mM Tris-HCl, 20 mM ATP and 0.06 mM of $MgSO_4$, pH 7.5; the reaction was stopped by the addition of 0.2 mM EDTA. 3, factor F_1 was incubated in conditions similar to those in the experiment (1), but at a pressure of 150 atm. 4, factor F_1 incubated in conditions similar to those in the experiment (2) at a pressure of 150 atm.

the reaction rate on the substrate concentration for factor F_1 modified with dimethylsuberimidate obeyed Michaelis-Menten kinetics, with $K_{m,app}$ equal to 0.3 mM (Fig. 7, curve 2). Factor F_1 treated with dimethyladipimidate did not differ from the native enzyme in its catalytic properties (data not shown). Factor F_1 preincubated with Mg^{2+} is subject to Michaelis-Menten kinetics for the entire interval of substrate concentration studied (0.01—2 mM), with $K_{m,app}$ equal to 0.04 mM and V equal to 15 μmol/min per mg protein (Fig. 7, curve 3).

Preincubation with $MgSO_4$ did not change the kinetic parameters of factor F_1 treated with dimethylsuberimidate (not shown). But factor F_1 which was first preincubated with $MgSO_4$ and then treated with dimethylsuberimidate hydrolysed ATP with the same $K_{m,app}$ as did the enzyme preincubated with

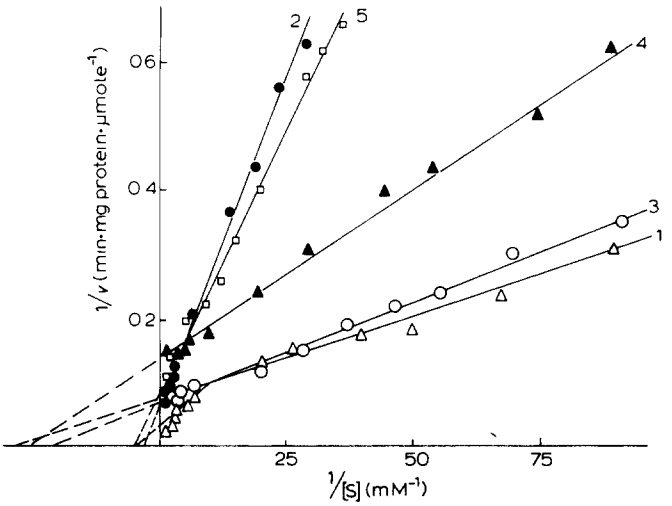


Fig. 7. Changes in the parameters of the ATPase reaction as a result of the modification of factor F_1. Curve 1, control preparation of factor F_1. Curve 2, factor F_1 treated with dimethylsuberimidate (for the conditions of treatment, see the caption to Fig. 1). Curve 3, factor F_1 (1 mg/ml) preincubated with 1 mM $MgSO_4$ for 60 min. After 60 min 2 mM EDTA was added to the preincubation medium. Curve 4, factor F_1 was preincubated with 1 mM $MgSO_4$ and 50 mM $(NH_4)_2SO_4$ for 60 min, then 2 mM EDTA and 1% dimethylsuberimidate were added in 100 mM Hepes buffer, pH 8.5, and incubated for another hour. Curve 5, control preparation of factor F_1 with Mg-GTP as the substrate. The ATPase activity was measured in an ATP-regenerating system (see caption to Table II). The concentration of the Mg-ATP complex was calculated using a dissociation constant of $5 \cdot 10^5$ M [24].

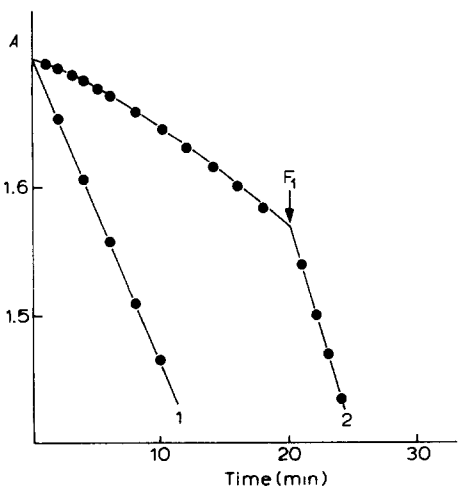


Fig. 8. The reactivation of factor F_1 preincubated with 5′-adenylyl imidodiphosphate. Curve 1, control preparation of factor F_1. Curve 2, factor F_1 (1 mg/ml) was preincubated for 10 min with 1 mM $MgSO_4$ and 10 μM 5′-adenylyl imidodiphosphate. The reaction was started by adding 1 μl of the solution of factor F_1 to the medium to measure ATPase activity (final volume 2 ml). The composition of the measurement medium was as given in the caption to Table II, but the concentration of Mg-ATP was 0.025 mM. The moment at which 1 μg of the control preparation of factor F_1 was added is indicated by an arrow.

$MgSO_4$ but not subjected to cross-linking by dimethylsuberimidate (Fig. 7, cruves 3 and 4).

The dependence of the Mg-GTP hydrolysis rate on substrate concentration obeyed Michaelis-Menten kinetics throughout the range of the substrate concentration studied with $K_{m,app} \approx 0.2$ mM and $V \approx 12.5$ μmol/min per mg protein (Fig. 7, curve 5).

We also studied the interaction of factor F_1 with the non-hydrolysable analog of ATP, 5′-adenylyl imidodiphosphate . Factor F_1 preincubated with 10 μM 5′-adenylyl imidodiphosphate had an ATPase activity close to zero which was slowly increased after 1000-fold dilution (Fig. 8). The reactivation process was subject to the equation of a monomolecular reaction, and was evidently the result of the slow dissociation of 5′-adenylyl imidodiphosphate from the enzyme. The rate constant of reactivation was determined from the plots $\ln(1 - (V_t/V))$ on t, where V_t is the reaction rate at time t, and V is the maximal reaction rate which is demonstrated by factor F_1 after the complete release of 5′-adenylyl imidodiphosphate. This constant is equal to $2 \cdot 10^{-2}$ min^{-1}. Treatment of factor F_1 with dimethylsuberimidate did not prevent the binding of 5′-adenylyl imidodiphosphate with the enzyme and did not change the dissociation rate of the complex of factor F_1 with 5′-adenylyl imidodiphosphate (not shown).

Discussion

Treatment of factor F_1 with 1% dimethylsuberimidate in the presence of 50 mM $(NH_4)_2SO_4$ leads to the formation of four different types of covalently cross-linked dimers of the subunits: αα, αβ, αγ, βγ, on average one dimer per molecule of the enzyme (Fig. 1). Such modification results in 60—70% inactivation of the enzyme. The cross-linked enzyme did not show a change in the sedimentation coefficient and was not inactivated in the cold (Table I).

Thus, the cross-linking of any of these pairs of subunits (αα, αβ, αγ, βγ) in a molecule of factor F_1 results in the protection of the enzyme against the cold. Such a situation is only possible if one of the stages of cold inactivation includes a co-operative rearrangement of the quaternary structure of the enzyme during which a change in the mutual distribution of all the subunits occurs. In actual fact, according to the data of Penefsky and Warner [16], the first stage in the cold inactivation of factor F_1 consists of a conformational rearrangement of the protein molecule which is accompanied by a drop in the sedimentation coefficient from 12 S to 9 S. At the following stage the dissociation of the 9-S form of the protein into subunits occurs. Experiments on the sedimentation of factor F_1 treated with dimethylsuberimidate and subjected to incubation in the cold in the presence of 0.5 M KCl have shown that the enzyme retains a sedimentation coefficient of 12 S. Consequently, the formation of a cross-link blocks the 12 S → 9 S conformational change, and, thus, prevents the inactivation of the enzyme.

The use of bifunctional cross-linking reaagents is a helpful approach to elucidating the nature of the conformational rearrangements in factor F_1 under the effect of some effectors. Dimethylsuberimidate abolishes the activating effect of certain anions, HCO_3^-, SO_3^{2-}, and inactivation of factor F_1 preincubated with

Mg^{2+} (Table I). These results indicated that the mechanism of action of anions an Mg^{2+} involves conformational rearrangements in the quaternary structure of the enzyme. Conformational changes induced by Mg^{2+} were registered by us previously, using spin-labelled probes [30]. It is interesting that the new conformation of the enzymes that emerges under the effect of Mg^{2+} differs from the initial one in its kinetic parameters and may, apparently, be fixed by dimethylsuberimidate (Fig. 7).

The treat ment of factor F_1 with dimethyladipimidate does not lead to intersubunit cross-linking and does not inactivate the enzyme or stabilise it (Figs. 1, 2, Table I). Neither cross-linking of the subunits nor changes in the properties of factor F_1 occur if it is successively treated with dimethyladipimidate and then dimethylsuberimidate. Thus, the formation of intersubunit cross-linking is a result of the reaction between dimethylsuberimidate and the amino groups, the distance between which is more than 8 Å, but does not exceed 11 Å. It is the formation of such cross-links and not the modification of those very amino groups which apparently causes the change in the properties of factor F_1.

Dimethylsuberimidate also blocks the structural rearrangements in factor F_1 that are induced by tight binding of ADP and cuase inactivation of the enzyme although the ability to bind ADP tightly is retained (Table II). The tight binding of ADP followed by inactivation has also been observed in other studies [5].

It may be suggested that the ADP-induced inhibition of ATPase includes two stages: the binding of ADP in the tight binding site, and the subsequent conformational change, which is accompanied by inactivation and blocked by dimethylsuberimidate. The occurrence of these structural rearrangemens in factor F_1 as a result of ADP binding was demonstrated by us earlier, using hydrophobic spin probes [30]. Since dimethyladipimidate, unlike dimethylsuberimidate, has no effect on the inhibition of factor F_1 by ADP (Table II), it may be suggested that ADP induces rearrangements in the quaternary structure of factor F_1.

The overall number of ADP molecules tightly bound to a molecule of factor F_1 treated with dimethylsuberimidate is five (Table II). This result demonstrates that factor F_1 has at least six nucleotide-binding sites (five tight ADP-binding sites and a catalytic site). This conclusion is also confirmed by the results of experiments in which ADP binding to factor F_1 measured in the presence of inorganic phosphate (Table II).

The hydrolysis of Mg-ATP and Mg-GTP by isolated factor F_1 is accompanied by slow inactivation, which is caused neither by ADP, Mg^{2+} nor by the accumulation of any other competitive inhibitors, but is determined by the slow conformational change accompanying substrate hydrolysis (Fig. 3). Similar results were published recently by Dr. R.B. Beechey and co-workers [17]. The inactivation rate constant (k) increases as the concentration of the substrate grows. The observed character of the dependence of $1/k$ on $1/[S]$ (Fig. 4) is reminiscent of the dependence of the inverse initial rate of hydrolysis on the inverse substrate concentration (Fig. 7). The two processes are characterised by similar $K_{m,app}$ values. Therefore, one and the same intermediate enzyme-substrate complex takes part in two processes: substrate hydrolysis and inactivation. The more turnovers of the enzyme, the greater is the probability of inactivation. This is in good agreement with the finding that the continual presence

of the substrate is needed for inactivation to occur. The experiments with 7-chloro-4-nitro-2-oxa-1,3-diazole showed that the binding of the substrate without its hydrolysis (see Ref. 27) does not lead to inactivation. The probability of inactivation per turnover of the enzyme is equal to $2 \cdot 10^{-6}$ for the entire interval of the Mg-ATP concentration studied (2—0.025 mM). Thus, the mechanism of inactivation does not depend on whether the second nucleotide-binding site of factor F_1 is saturated or not (see below). It is interesting to note that for Mg-GTP hydrolysis the probability of inactivation per turnover is $2 \cdot 10^{-5}$, i.e. it is one order of magnitude higher than for the hydrolysis of Mg-ATP.

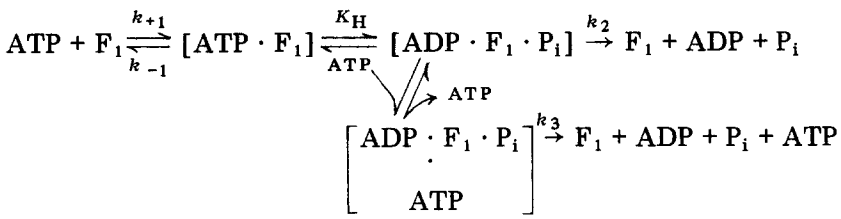
Dimethylsuberimidate does not affect the binding of 5′-adenylyl imidodiphosphate with factor F_1 (Fig. 8). ATPase activity is only partially inhibited by dimethylsuberimidate (Fig. 2, curve 2). Thus, the binding of the substrate and also the very hydrolysis of ATP and the dissociation of the reaction products do not necessarily involve major co-operative rearrangements in the quaternary structure of the enzyme. On the other hand, ATP-induced inactivation of facto F_1 is apparently coupled with the co-operative rearrangement of the quaternary structure, since the formation of one intersubunit bond in a molecule of factor F_1 by dimethylsuberimidate completely prevents inactivation (Fig. 3, curve 2). This rearrangement occurs with a probability of approximately one event to $5 \cdot 10^5$ catalytic acts.

An interesting feature of the structural rearrangement in the process of ATP-induced inactivation of factor F_1 is that this process is accelerated by moderate pressure (Fig. 3). This indicates that one of the stages of inactivation of factor F_1 is accompanied by a lowering of the partial specific volume of the enzyme. The changes in the partial specific volume ($\Delta V^* = 5 \cdot 10^{-4}$ cm^3/g) are 0.07% of the initial value ($V = 0.74$ cm^3/g [4]). This decrease in the partial specific volume must be connected with the considerable change in the degree of hydrophobicity of the surface of the protein molecule.

Another type of conformational change induced by ATP determined the complex non-Michaelian kinetics of ATP hydrolysis by factor F_1 (Fig. 7, see also Refs. 31, 32). In understanding the meaning of the differences in the parameters of the ATPase reaction at low and high concentrations of the substrate, the data of Choate et al. [33] are of great value. From their results it follows that at very low concentrations of ATP a dynamic equilibrium ATP + $H_2O \rightleftharpoons$ ADP + P_i is established in the active site, and the ADP phosphorylation rate is higher than that of ADP and P_i release into water. Thus, the ATPase activity is limited by the steps of the products' release. If the concentration of ATP was increased to 10—20 μM, the ADP phosphorylation rate became less than that of ADP release into the solution. The binding of a second molecule of ATP at a high substrate concentration (0.1—2 mM) does, apparently, result in a several-fold increased rate of ADP and P_i release. Since the rates of ATP synthesis and hydrolysis in the active site are similar (the equilibrium constant of the hydrolysis is not more than 10, see below), it may be assumed that at a high ATP concentration the hydrolysis of the substrate in the active site is the limiting step of the ATPase reaction.

It may be suggested that the mechanism of the ATPase reaction can be

described by the scheme:



The ATP binding to the active site is almost irreversible; the rate constant of dissociation of the $[F_1 \cdot ATP]$ complex, k_{-1}, is equal to $2.1 \cdot 10^{-2}$ min^{-1}. This constant was measured as the rate of the ATPase reactivation as a result of 5′-adenylyl imidodiphosphate release, assuming that 5′-adenylyl imidodiphosphate was bound to the active site.

A second molecule of ATP is bound at high concentrations of the substrate, and this results in an increase in the rate of release of ADP and P_i from the active site of factor F_1 ($k_3 > k_2$). At the same time, the binding of the second ATP molecule to factor F_1 decreases the affinity of the active site to the substrate, Mg-ATP ($K_{m,app}$ at a high ATP concentration increases up to 0.3 mM, Fig. 7, curve 1). Thus, both the changes in the kinetics of ATP hydrolysis at a high substrate concentrations (the increase in the rate and the increase in $K_{m,app}$) can be explained by a lowering of the affinity of the catalytic site of factor F_1 to ATP and ADP as a result of the second ATP molecule binding to the enzyme.

In the opinion of Choate et al. [33] two active sites work alternately in isolated factor F_1; the binding of the substrate to one site increases the rate at which the products are released from the other. An alternative explanation of the results observed is that ATP hydrolysis occurs in one active site and the acceleration of ADP and P_i release is the result of the binding of a second molecule of ATP to the non-catalytic site. The latter suggestion is in good agreement with a number of facts. (1) The dependence of the hydrolysis rate of GTP, which poorly binds with the non-catalytic site [2,6], on the concentration of the substrate, obeys Michaelis-Menten kinetics (Fig. 7, curve 5). (2) Complete inhibition of both the ATPase and the GTPase activity of factor F_1 by the mixed anhydride of ATP and mesitylenercarboxylic acid is achieved as a result of the binding of one molecule of the inhibitor to the enzyme [28,29]. (3) The partial inhibition of factor F_1 by this inhibitor does not change the character of the dependence of ATPase activity on the substrate concentration.

The formtion of one cross-linked dimer of the subunits in a molecule of factor F_1 by dimethylsuberimidate or the conformational rearrangement induced by Mg^{2+} disturbs the allosteric interaction between two ATP-binding sites (or prevents the binding of a second molecule of ATP) (Fig. 7). The values of $K_{m,app}$ differ after this treatment, and this indicates the different mechanism of action of dimethylsuberimidate and Mg^{2+}.

At low concentrations of Mg-ATP (i.e. when the binding of the second molecule of ATP does not occur) the hydrolysis reaction takes place with $K_{m,app}$ equal to 0.03 mM and k_{cat} equal to $5 \cdot 10^3$ min^{-1} (Fig. 7, curve I). In this case, $k_{+1} = k_{cat}/K_{m,app}$ is equal to $1.7 \cdot 10^8$ $M^{-1} \cdot min^{-1}$. The constant of ATP disso-

ciation from the active site of ATPase $K_{ATP} = k_{-1}/k_{+1}$ is equal to approx. 10^{-10} M. The ADP binding constant does apparently coincide with the constant of competitive inhibition of ATPase by ADP and is $1.5 \cdot 10^{-4}$ M [34,35]. We have no data allowing us to determine precisely the equilibrium constant for the binding of P_i in the active site of factor F_1. The fact, however, that P_i in a concentration of 10 mM does not inhibit the ATPase reaction indicates that the value of this constant is more than 10^{-2} M. By comparing the data on the binding constants of ADP and P_i in the active site with the values of the ATP binding constant obtained above, it may be concluded that the difference in the energies of binding of ATP and of the ATPase reaction products can ensure the reversibility of ATP hydrolysis at the active site of ATPase. K_H does not exceed 10.

A similar conclusion may be drawn on the basis of the following independent consideration. As already noted above, the rate of ADP phosphorylation in the active site of factor F_1 is greater than the ADP and P_i release rate from the active site at low concentrations of ATP in solution [33]. Considering that the release of ADP and P_i from the active site is the rate-limiting step of the ATPase reaction [33], and also the data on the ATPase reactions rate, it may be concluded that the rate of ATP synthesis at the active site is more than 10^3 turnovers per min. On the other hand, the maximum rate of ATP hydrolysis, like that of any other hydrolase reactions occurring according to the mechanism of acid-base catalysis, should not exceed 10^5 turnovers per min. * The ratio of the maximum possible rate of ATP hydrolysis at the active site to the minimum rate of synthesis results in a value of K_H of not more than 100.

The data on the dissociation rates of the complex of factor F_1 with ADP and P_i ($k_2 = 5 \cdot 10^3$ min^{-1}) and with 5'-adenylyl imidodiphosphate ($2 \cdot 10^{-2}$ min^{-1}) demonstrate that the release of ATP from the active site of factor F_1 takes place approx. 10^5-times more slowly than the release of ADP and P_i. Thus, the differences in the energies of binding of ATP and (ADP + P_i) are evidently determined by the difference in the dissociation rates of the corresponding enzyme-substrate complexes.

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* The given value (10^5 turnovers per min) is the limit for the substrate protonation rate at the active site of the enzyme by a proton-donating group during acid-base catalysis. In actual fact, none of the known enzymes possessing hydrolytic activity works at a higher rate.

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