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THE INTERACTION OF MITOCHONDRIAL F_1-ATPase WITH THE NATURAL ATPase INHIBITOR PROTEIN

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Summary

The interaction of soluble mitochondrial ATPase from beef heart with the natural ATPase inhibitor was studied. It was found that the phosphorylation of small amounts of ADP by phospho*enol*pyruvate and pyruvate kinase, and an ensuing catalytic cycle supports the binding of the inhibitor to the enzyme. The association of the inhibitor with F_1-ATPase does not increase the content of ATP in the F_1-ATPase-inhibitor complex. The inhibitor of catalytic activity bathophenanthroline-Fe^{2+} chelate prevents the interaction, while the association of the inhibitor with F_1-ATPase is delayed if the reaction is carried out in ^2H_2O. The data indicate that a transient state involved in the catalytic cycle is the form of the enzyme that interacts with the inhibitor.

The proton-motive force-induced dissociation of the inhibitor from particulate ATPase is prevented by bathophenanthroline-Fe^{2+} chelate and nitrobenzofurazan chloride, which indicates that a functional catalytic (β) subunit is required for the proton-motive force-induced release of the inhibitor. The data suggest a direct involvement of catalytic (β) subunit in the mechanism by which the F_1-ATPase senses the proton-motive force.

Introduction

A protein that inhibits the hydrolytic activity of soluble and particulate mitochondrial ATPase was originally isolated from beef heart mitochondria by

Abbreviations: EDTA, ethylenediaminetetraacetate; FCCP; carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mes, 2-(*N*-morpholino)ethanesulfonic acid; F_1-ATPase, soluble, oligomycin-insensitive mitochondrial ATPase.

Pullman and Monroy [1]. The protein has also been isolated from yeast mitochondria [2,3], chloroplasts [4], bacteria [5] and liver [6]. The protein was found to inhibit ATP-supported reactions of mitochondria, and was thus proposed to exert a regulatory role on mitochondrial functions [7]. In more recent work, it has been shown that the protein also inhibits photophosphorylation in chloroplasts [8] and oxidative phosphorylation in submitochondrial particles [9,10]. The experimental data showed that the inhibitor is displaced from the F_1 component of the ATPase complex upon the establishment of a proton-motive force [9], and that this dissociation induces a transition of the ATPase to an active state in which steady-state phosphorylation is attained. Accordingly, knowledge of the factors that control the association and dissociation of the inhibitor with the soluble and particulate component is of interest, since it may shed light on the mechanism through which F_1 'senses' the proton-motive force and catalyzes the synthesis of ATP.

This paper describes a study of the events that occur during the association and dissociation of the inhibitor with soluble and particulate F_1. The results indicate that the enzyme undergoes conformational changes which may be monitored by the displacement of the inhibitor, and that these changes may well be the primary effect of the proton-motive force on particulate F_1 in the process through which the enzyme catalyses the synthesis of ATP.

Material and Methods

Mg-ATP particles from bovine heart mitochondria were prepared as described elsewhere [11]. Soluble F_1 [12], and the ATPase inhibitor [13] were likewise prepared by established procedures. ATPase activity was measured in the presence of an ATP regenerating system [14] by recording NADH oxidation at 340 nm, or by assay of inorganic phosphate formed during ATP hydrolysis [15]; the experimental conditions are detailed under Results. In several of the experiments, it was necessary to remove small molecular weight compounds from soluble F_1; this was achieved by filtering the solutions through syringes that contained Sephadex G-50-Fine (equilibrated with 25 mM Tris acetate, pH 7.4) as described by Penefsky [16]. ATP was assayed by the luciferin-luciferase assay [17]; all determinations of the emitted light were carried out at least in quintuplicate.

Results

Association of F_1-ATPase with inhibitor

It has been reported that the binding of the natural ATPase inhibitor to F_1 is promoted by Mg-ATP [18]. More recently, Gómez-Fernández et al. [19] presented evidence indicating that the rate at which the inhibitor interacts with F_1 depends on the rate at which ATP (or analogs) are hydrolyzed. In agreement with the latter experiments, the data of Table I show that the phosphorylation of very low amounts of residual ADP that remain in F_1 passed through Sephadex (as detected by the amount of radioactive adenine nucleotides that remain after filtration) by phospho*enol*pyruvate and pyruvate kinase and an ensuing catalytic cycle supports the interaction of F_1 with inhibitor. Under these con-

TABLE I

BINDING OF INHIBITOR TO SOLUBLE F_1-ATPase AS SUPPORTED BY Mg-ATP AND PYRUVATE KINASE

To F_1 (200 μg) which had been stored in 50% saturated $(NH_4)_2SO_4$ (pH 7.4) centrifuged, and dissolved in 200 μl 25 mM Tris acetate, 100 pmol of carrier-free [8-^{14}C]ATP (11 580 dpm) were added and the solution was allowed to stand for 15 min. Subsequently, the solution was filtered through Sephadex syringes (see Methods), whiche eliminated residual $(NH_4)_2SO_4$ and non-bound adenine nucleotides. An aliquot of the filtrate was withdrawn for assay of remaining adenine nucleotides by scintillation counting. A total of 84 dpm were recovered. The filtered enzyme (3 μg) was incubated (where shown) in 25 μl of 20 mM Tris-Mes (pH 6.5), 4 mM phospho*enol*pyruvate (PEP) and 5 units of pyruvate kinase (PK) (desalted), (PEP + PK), 1 mM KCl, 1 mM $MgSO_4$ and 0.3 μg inhibitor. After 10 min 1 ml of 25 mM Tris acetate (pH 7.4), 3 mM ATP and 3 mM magnesium acetate was added in order to assay ATPase activity. The reaction was stopped with trichloroacetic acid and inorganic phosphate determined. Where shown, no addition, or 1 mM Mg-ATP instead of phospho*enol*pyruvate and pyruvate kinase was added to the mixture in which binding of the inhibitor was studied. The hydrolytic activity of F_1 in the incubation mixture at pH 6.5 was determined in an identical mixture, except that it was scaled to 3 ml and 0.6 mM NADH and 2 units of lactate dehydrogenase were added to the mixture that contained phospho*enol*pyruvate and pyruvate kinase; to this cuvette no ATP was added. To the mixture with ATP, phospho*enol*pyruvate and pyruvate kinase were also added. Oxidation of NADH was taken as an index of ATP hydrolyis. The numbers indicate μmol ATP hydrolyzed per min per mg.

Additions to incubation mixture at pH 6.5	Hydrolysis at pH 6.5	Hydrolyis at pH 7.4	
		no inhibitor	+inhibitor
—	0	57	41.0
Mg-ATP	9.4	58	1.6
PEP + PK	0.24	68	6.0

ditions the rate of ATP hydrolysis was extremely low, about 0.2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ F_1.

These results indicate that bound ADP may be converted by pyruvate kinase to ATP, and that this condition induces the binding of inhibitor to F_1. The following experiments were designed to explore the mechanisms involved in the interaction.

If the form of F_1 that interacts with the inhibitor is the enzyme that contains ATP, it would be expected that the content of ATP per F_1 would increase upon binding of the inhibitor. Accordingly the amount of ATP in the F_1-inhibitor complex was measured and compared to that of an enzyme which had been treated under identical conditions in the absence of inhibitor. Various preparations of F_1 with different ATP contents and specific activities were used to explore whether the binding of the inhibitor protein to F_1-ATPase raised the content of ATP in the F_1-inhibitor complex to values above 1. The results showed (Table II) that the content of ATP in F_1 did not increase in a significant manner when the enzyme underwent interaction with the inhibitor. This observation suggests that it is not an ATP containing form of F_1, as such, that is specifically required for the binding of the inhibitor. It would rather appear that the catalytic cycling of the enzyme involves the transient appearance of a conformational state that is required for the binding of the inhibitor. It follows from this conclusion that any agent which interferes with the catalytic cycle of the enzyme would inhibit the binding of the inhibitor.

Inhibition of the hydrolytic activity of F_1 by bathophenanthroline-Fe^{2+} chelate [20] prevented, as expected, the interaction of F_1 with inhibitor (Table

TABLE II

CONTENT OF ATP IN F_1-INHIBITOR COMPPLEX

F_1 (5 mg) was incubated with and without inhibitor (500 μg) in 2.0 ml of 20 mM Tris-Mes (pH 6.5) 0.5 mM Mg-ATP for 20 min. At this time, in all experiments the activity was inhibited more than 90% by the inhibitor. The mixture was filtered through Sephadex syringes. In control experiments under identical conditions without F_1 and inhibitor, filtration removed more than 99% of the nucleotides as assay by filtration of [8-^{14}C ATP]. Aliquots of filtered F_1 and F_1-inhibitor complex were removed for assay of enzymatic activity. F_1 continued to be inhibited by the inhibitor by about 80% in all experiments. Aliquots were also withdrawn and boiled immediately after filtration and used for assay of ATP by the luciferin-luciferase assay [16]. ATP content was not modified by boiling as determined by recovery experiments. Determinations of ATP were made at least in quintuplicate.

Experiment	Specific activity of starting F_1	mol ATP/mol F_1	
		Free	F_1-inhibitor complex
1	60	0.38	0.53
2	32	0.33	0.55
3	72	0.86	1.04
4	51	1.10	0.9

III). The effect was relieved by FCCP, which reverses the bathophenanthroline-Fe^{2+} chelate induced inhibition of ATP hydrolysis by F_1.

It has been reported [21] that the ATPase activity of soluble F_1 is decreased by incubation of the enzyme in media which contains ^2H_2O in comparison to the activity measured in H_2O. The lower activity of the enzyme in ^2H_2O results from the stronger interaction between the enzyme and the solvent which retards the conformational change that the enzyme undergoes when it is carrying out ATP hydrolysis [21]. Fig. 1 shows that the interaction of soluble F_1-ATPase with the ATPase inhibitor is significantly delayed if the interaction is carried out in 82% ^2H_2O.

Apparently the delay in the interaction of F_1-ATPase with the inhibitor is not due to a lower ATPase activity in ^2H_2O (20% lower in ^2H_2O; see legend of Fig. 1), since Gómez-Fernández et al. [19] showed that more drastic decreases of activity are required to diminish the rate of interaction with the inhibitor. Moreover the data of Table I show that at rates of about 2% of the control (at

TABLE III

EFFECT OF BATHOPHENANTHROLINE-Fe^{2+} CHELATE ON THE BINDING OF INHIBITOR TO F_1

Preincubation was performed in the presence of ATP + Mg^{2+} as described in Table I. 34 μg ATPase inhibitor/mg F_1 was used. Where shown the preincubation mixture also contained 10 μM bathophenanthroline-Fe^{2+} chelate ($(Bph)_3Fe^{2+}$) and 1 μM FCCP.

Additions to preincubation	ATPase activity (μmol/min/mg protein)	
	—FCCP	+FCCP
F_1	34.5	—
F_1 + inhibitor	5.4	3.8
F_1 + $(Bph)_3Fe^{2+}$	1.6	16.8
F_1 + inhibitor + $(Bph)_3Fe^{2+}$	4.3	15.7
F_1 + inhibitor + $(Bph)_3Fe^{2+}$ + FCCP	6.1	—
F_1 + inhibitor + FCCP	6.2	—

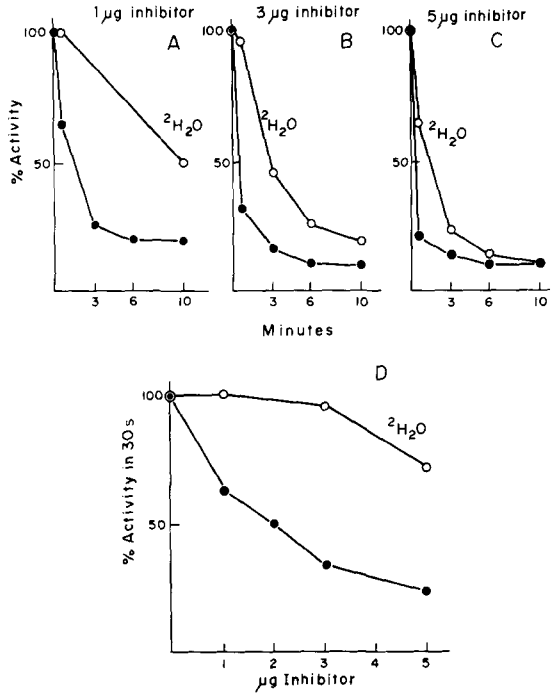

Fig. 1. Effect of ^2H_2O on the binding of the inhibitor to soluble F_1. Soluble F_1 (16 μg) was incubated with the indicated amounts of inhibitor in 20 mM Tris-Mes (pH 6.5) and 2.5 mM Mg-ATP in 100% H_2O or in 82% ^2H_2O in a final volume of 200 μl. At the times shown aliquots were withdrawn for the measurement of ATPase activity in all water medium. The specific activity of F_1-ATPase at pH 6.5 was 12.3 whilst in ^2H_2O it was 9.8. In D the incubation time was 45 seconds.

pH 6.5), total interaction of inhibitor with F_1 is observed in a period of 10 min, whilst in ^2H_2O, total interaction of F_1 and inhibitor is not attained in the same time (Fig. 1). Most likely, the delay in the time of interaction between inhibitor and F_1 caused by ^2H_2O is due to an increase in the hydrophobic interactions within F_1 [21] required for the binding of the inhibitor [19].

These findings, together with those of Gómez-Fernández et al. [19] are consistent with the idea that during its catalytic cycle, F_1 undergoes a change in conformation, and that, in the transitional configuration, F_1 interacts with the inhibitor.

Ferguson et al. [22] have shown that the adenine nucleotide analog 4-chloro-7-nitrobenzofurazan binds covalently to a tyrosine residue of a β-subunit, and that this results in total inhibition of hydrolytic activity. According to the results of these authors and those of Lunardi and Vignais [23], the tyrosine residue is at the catalytic site of the β-subunit of F_1. Therefore, a study of the interaction of the F_1-inhibitor complex with chloronitrobenzofurazan may provide information concerning the relationship between the catalytic site and the inhibitor binding site of the enzyme.

Soluble F_1-ATPase was incubated with the natural ATPase inhibitor; the ATPase activity was inhibited 96%. An aliquot of the latter solution was incubated with chloronitrobenzofurazan, and subsequently filtered through Sephadex syringes to remove free chloronitrobenzofurazan (Fig. 2). It is to

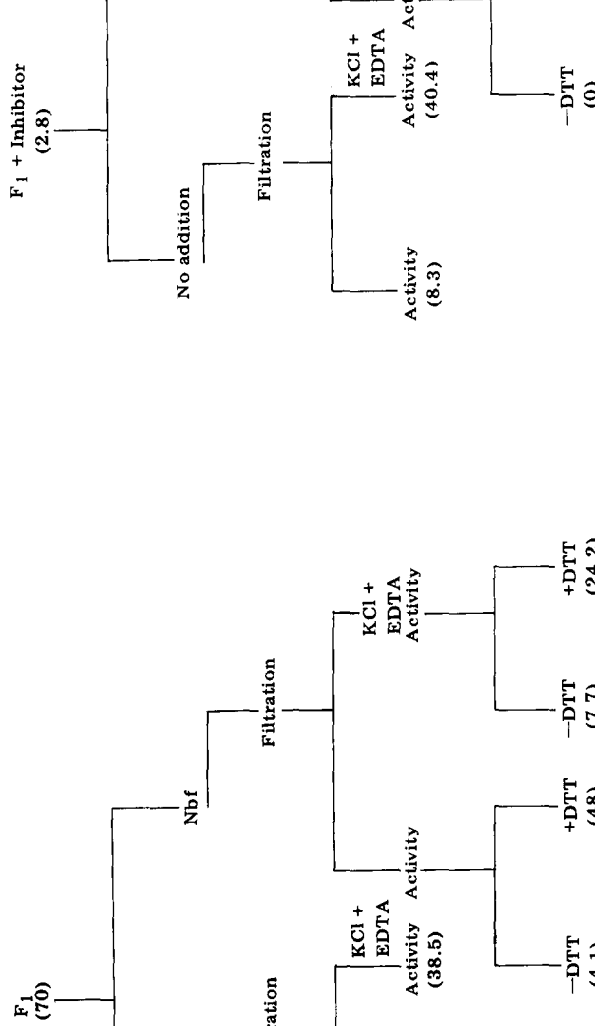

Fig. 2. Binding of 4-chloro-7-nitrobenzofurazan (Nbf) to the F_1-inhibitor complex. F_1 (46 μg) were incubated in 210 μl of 7.5 mM Tris-Mes (pH 6.5), 5 mM Mg-ATP and 10 μg inhibitor for 10 min at 30°C. In the control the inhibitor was omitted. After 10 min of incubation the activity of the fraction was recorded; the ATPase activity was inhibited 96% by the inhibitor. 150 μl of 250 mM Tris acetate (pH 7.4) and 1 mM EDTA were added and two aliquots of 175 μl were taken out from each test tube, to one of them 2 μl of 10 mM Nbf (in ethanol) was added; to the other 2 μl ethanol was added. The mixture was incubated for 2 h and thereafter passed through Sephadex syringes. The ATPase activities of the filtrates were measured. To the rest 50 μl of 2.0 M KCl, 25 mM Tris acetate (pH 7.4) and 5 mM EDTA were added to induce dissociation of the inhibitor F_1. An aliquot of those fractions was withdrawn for measurements of ATPase activity in the absence and presence of dithiothreitol (DTT). The numbers in parenthesis indicate the activity of the samples in μmol/min/mg.

be noted that under these conditions chloronitrobenzofurazan does not induce dissociation of the F_1-inhibitor complex, since very low ATPase activity was detected even in the presence of dithiothreitol which effectively breaks the covalent bond between chloronitrobenzofurazan and the enzyme (Ref. 22, and control experiments of Fig. 2).

In a subsequent step of the experiment, the F_1-inhibitor complex was incubated with high concentrations of salt and EDTA, and its dissociation was induced; significant loss of enzymatic activity was observed in this step. However in the case of the chloronitrobenzofurazan-treated complex, the preparation that results from the latter treatment is an enzyme with an ATPase activity that becomes apparent only in the presence of dithiothreitol. Therefore the results of Fig. 2 indicate that the F_1-inhibitor complex contains a site that is capable of interacting with the adenine nucleotide analog chloronitrobenzofurazan.

Dissociation of the F_1-inhibitor complex in Mg-ATP particles by electrochemical gradients

If the inhibitor interacts with an intermediate state of F_1 that appears during active catalysis, it would be expected that the proton-motive force induced dissociation would involve a conformational change which would also be related to the catalytic process. Therefore, it was of interest to explore whether electrochemical gradients could induce dissociation of particulate F_1-inhibitor complex, under conditions at which conformational changes that occur during catalytic activity are prevented.

For this purpose, Mg-ATP particles were incubated with bathophenanthroline-Fe^{2+} chelate and subsequently exposed to an electrochemical gradient as generated by NADH oxidation (Table IV). NADH oxidation was not ostensibly affected by this concentration of bathophenanthroline-Fe^{2+} chelate. After exposure to NADH oxidation, which leads to a removal of the inhibitor [9,24], ATPase activity was assayed in the presence of FCCP to relieve the effect of bathophenanthroline-Fe^{2+} chelate on F_1 [20,25].

The results of Table IV indicate that bathophenanthroline-Fe^{2+} chelate prevents the electrochemical potential induced dissociation of the F_1-inhibitor

TABLE IV

EFFECT OF BATHOPHENANTHROLINE-Fe^{2+} CHELATE ON THE PROTON-MOTIVE FORCE INDUCED ASSOCIATION ON INHIBITOR FROM Mg-ATP PARTICLES

Mg-ATP particles (86 μg) were incubated for 2 min in 1 ml of 25 mM Tris acetate (pH 7.4), 3 mM magnesium acetate, and 25 μM bathophenanthroline-Fe^{2+} chelate ($(Bph)_3Fe^{2+}$) were shown. At this time 0.6 mM NADH was added also where shown. Three minutes after the addition of NADH, 3 mM ATP and 30 μM FCCP were added to initiate ATP hydrolyisis and to revert the action of $(Bph)_3Fe^{2+}$. Hydrolysis was allowed to proceed for 5 min at which time the reaction was stopped and inorganic phosphate determined.

Additions to preincubation mixture	ATPase activity (μmol/min/mg)
—	0.88
$(Bph)_3Fe^{2+}$	0.72
NADH	2.07
$(Bph)_3Fe^{2+}$ + NADH	0.98

TABLE V

EFFECT OF 4-CHLORO-7-NITROBENZOFURAZAN (Nbf) ON THE ACTIVATION OF ATPase OF Mg-ATP PARTICLES BY A PROTON-MOTIVE FORCE

Mg-ATP particles (2 mg of protein) were incubated in 200 μl of 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5) and 200 μM Nbf where shown for 60 min at 4° C, these particles are referred to as Mg-ATP-Nbf. Measurements of ATPase activation were carried out as follows: Control Mg-ATP particles (100 μg protein) were incubated with 6 mM rotenone (to allow diffusion of rotenone and thereby limit the aerobic oxidation of NADH) in 3 ml of 180 mM sucrose, 30 mM potassium acetate, 3 mM $MgSO_4$, and 3 mM magnesium acetate for 5 min. At this time activation of ATPase was induced by adding 0.2 mM NADH and its oxidation was recorded for 8 min. Subsequently, the concentration of rotenone was raised to 1 μM to completely inhibit the aerobic oxidation of NADH and ATPase activity was recorded by simultaneously raising the concentration of NADH to 0.2 mM and including in the mixture 3 mM ATP. Mg-ATP-Nbf particles were treated identically except that the addition of 6 nM rotenone was not made. After steady state ATPase activity was reached 1 μM FCCP followed by 1 mM dithiothreitol were added to the mixture and its effect on the activity recorded (+DTT).

Particles	ATPase (nmol/min/mg)		
	—	+FCCP	+DTT
Mg-ATP	183	274	279
Mg-ATP-Nbf	241	241	327
Mg-ATP + NADH	650	1928	1928
Mg-ATP-Nbf + NADH	602	1012	1205

complex. Since bathophenanthroline maintains F_1 in a fixed conformation (Ref. 25, and Hundal, T., Nordenbrand, K. and Ernster, L., unpublished results) as revealed by its protective effect on the enzyme against cold-dissociation, it would be seem that conformational changes are required not only for the interaction, but also for dissociation of the F_1-inhibitor complex.

More specifically, it would appear that the bathophenanthroline-Fe^{2+}-chelate sensitive site of F_1, which is on the β-subunit (Hundal, T., Nordenbrand, K. and Ernster, L., unpublished results), is involved in the mechanism by which F_1 senses the proton-motive force and releases the inhibitor.

Similarly to bathophenanthroline, chloronitrobenzofurazan bound to particulate F_1 also prevents the proton-motive force induced dissociation of the F_1-inhibitor complex. The experiment of Table V shows that the ATPase activity of Mg-ATP particles is increased about eight times by NADH oxidation (with FCCP from 241 to 1928 nmol · min^{-1} · mg^{-1}). In particles treated with chloronitrobenzofurazan, NADH oxidation induces a significant activation of the ATPase that is detected in the absence of dithiothreitol from 241 to 1012 nmol · min^{-1} · mg^{-1} in the presence of FCCP; this reflects the activity of the enzymes free of chloronitrobenzofurazan. The further addition of dithiotreitol to these particles induces only a small increment in ATPase activity over that obtained with FCCP (from 1012 to 1205 nmol · min^{-1} · mg^{-1}).

As the increment in activity induced by dithiothreitol reflects the activity of the enzymes that possessed covalently bound chloronitrobenzofurazan [22], and as the increment observed was relatively small, it may be inferred that the enzymes that possess chloronitrobenzofurazan fail to respond to NADH oxidation, otherwise a value close to 1928 nmol · min^{-1} · mg^{-1}, that of the control particles, should have been obtained.

With respect to the experiment outlines in Table V, several points should be

noted. First, the particles treated with chloronitrobenzofurazan are still coupled; this is concluded from the enhancement of ATPase activity induced by FCCP (from 602 to 1012 nmol · min^{-1} · mg^{-1} after exposure to NADH oxidation). Second, the activation of the ATPase in the control particles was carried out in the presence of a limiting amount of rotenone, so as to maintain similar respiratory rates in the two types of particles studied. Rotenone alone had no effect on the ATPase activity, in accordance with earlier findings [26]. Third, the ATPase activity of Mg-ATP particles is largely in the inactive state [9,10,24], and thus measurements of activity are not a reliable index to judge number of enzymes affected by chloronitrobenzofurazan; this explains why in Mg-ATP particles the effect of chloronitrobenzofurazan is apparently low; a better insight of the number of enzymes affected by chloronitrobenzofurazan may be obtained by comparing the activities in the absence of DTT of Mg-ATP and chloronitrobenzofurazan treated particles exposed to NADH (1928 and 1012 nmol · min^{-1} · mg^{-1}, respectively).

Discussion

The mechanism through which the F_1 component of the ATPase complex of mitochondria catalyzes the synthesis of ATP is still not settled. It has been proposed that the proton-motive force may be used initially for the formation of the covalent bond between ADP and P_i [27], or that it is utilized for the release of ATP [28,29] from F_1, with the simultaneous binding of ADP and P_i to a different but equivalent, alternate site [28]. In the latter mechanism, a proton-motive force-induced conformational change would be the driving force for ATP synthesis.

Recently it has been shown that proton-motive force induces the displacement of the ATPase inhibitor from the F_1 component of submitochondrial particles [9]. Since the dissociation of the inhibitor from F_1 involves a proton-motive force-induced conformational change of F_1-ATPase, it was considered that this dissociation could be utilized to monitor the sequence of events that occur in F_1 during the utilization of a proton-motive force in the formation of ATP from ADP and phosphate. However, it is first necessary to ascertain the characteristics of the F_1-inhibitor complex.

In the latter respect, the results of this work are in agreement with those of Gómez-Fernández et al. [19] who showed that during active hydrolysis, the enzyme interacts with the inhibitor. It is interesting that very low amounts of ADP suffice to induce inhibitor-F_1 interaction, provided an ATP regenerating system is introduced; thus it would appear that extremely low concentrations of ATP support the interaction. Moreover, the results may suggest that adenine nucleotide binding site(s) in equilibrium with added pyruvate kinase would be the site(s) involved in F_1-inhibitor interaction.

Under conditions in which the interaction of F_1 and inhibitor has been induced by Mg-ATP, it has been observed that the amount of ATP in the complex is not higher than that of the starting enzyme. On the other hand, bathophenanthroline-Fe^{2+} chelate, an inhibitor of the catalytic activity of F_1 [20], effectively prevented the binding of the inhibitor to F_1. Thus it would appear that some intermediate conformational state in the catalytic cycle of F_1, and

not the mere presence of ATP in the enzyme, is required for the interaction of the enzyme with the inhibitor.

^2H_2O delayed the interaction of the enzyme with the inhibitor, without a corresponding inhibition of the ATPase activity. Most likely, this effect of ^2H_2O is due to a stabilization of hydrophobic interactions in the enzyme that are required for inhibitor binding, in accordance with earlier observations [19].

It has been known for some time that energy supplied by electron transport induces a release of bound inhibitor from particulate F_1 [24,30], but it is only recently that direct evidence has been provided indicating that this effect is mediated by the proton-motive force generated by electron transport [9,10]. The present data show that the proton-motive force-induced release of the inhibitor from particulate F_1 is prevented by agents such as, bathophenanthroline-Fe^{2+} chelate and chloronitrobenzofurazan, which inhibit the ATPase activity of F_1, indicating that the process requires a catalytically active enzyme. Furthermore, since both bathophenanthroline-Fe^{2+} chelate (Hundal, T., Nordenbrand, K. and Ernster, L., unpublished results) and chloronitrobenzofurazan [22,23] interact with the β-subunit of F_1, which is generally considered to contain the catalytic site of the enzyme [31], our findings imply that a functional catalytic subunit is required for the proton-motive force-induced release of the inhibitor from F_1. These results strongly suggest that the catalytic (β) subunit is directly involved in the sensing of the proton-motive force by F_1. It is interesting in this connection that Chernyak and Kozlov [32] recently observed an apparent release of adenylyl imidotriphosphate from particulate F_1 under the influence of a proton-motive force. Since adenylyl imidotriphosphate represents a catalytically inert ATP analog, these data show that the proton-motive force acts on F_1 probably by inducing a release of ATP from its catalytic site, in accordance with the mechanism previously proposed [28,29].

Taken together with our findings, these results also suggest that the release of the natural inhibitor from F_1 as induced by the proton-motive force involves a simultaneous release of ATP from the catalytic site of the enzyme. In favor of this interpretation is the earlier finding [9,33] that ATP efficiently prevents the proton-motive force-induced release of the inhibitor. Future studies of the interaction of the naturally occurring inhibitor with F_1 may provide valuable information on the molecular mechanisms by which the catalytic site of F_1 senses the proton-motive force in the course of ATP generation.

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