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Inactivation of the mitochondrial ATPase inhibitor protein by chemical modification with diethylpyrocarbonate

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Modification of histidine residue(s) by diethylpyrocarbonate treatment of submitochondrial particles obtained by sonication results in inhibition of ATPase activity and stimulation of oligomycin-sensitive H^+ conduction. The inhibition of the ATPase (EC 3.6.1.3) activity persisted in F_1 isolated from diethylpyrocarbonate-treated submitochondrial particles, which exhibited the absorbance spectrum of modified histidine. Thus the inhibition of the ATPase activity results from histidine modification in F_1 subunits. Removal of the natural inhibitor protein from submitochondrial particles resulted in stimulation of proton conduction. After removal of F_1 inhibitor protein from the particles the stimulatory effect exerted by diethylpyrocarbonate treatment on proton conduction was lost. Reconstitution experiments showed that purified F_1 inhibitor protein lost, after histidine modification, its capacity to inhibit the ATPase activity and proton conduction. These observations show that the stimulation of proton conduction by the ATPase complex effected by diethylpyrocarbonate treatment results from histidine modification in F_1 inhibitor protein.

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; F_0, membrane integral sector of mitochondrial H^+-ATPase; F_1, catalytic part of mitochondrial H^+-ATPase; F_1 inhibitor protein, inhibitor protein of mitochondrial H^+-ATPase; EDTA-treated submitochondrial particles, submitochondrial particles prepared in the presence of EDTA; Sephadex-EDTA-treated submitochondrial particles, particles prepared by passing EDTA-treated submitochondrial particles through a Sephadex column and deprived of F_1 inhibitor protein; urea-treated submitochondrial particles, particles obtained by urea treatment of Sephadex-EDTA-treated submitochondrial particles.

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Introduction

The H^+-ATP synthase (F_0F_1-ATPase complex) of coupling membranes is made up of a hydrophobic sector (F_0) embedded in the lipid bilayer, that acts as a proton conductor during ATP synthesis or hydrolysis [1–6], and a hydrophilic sector (F_1) which contains the catalytic site(s) for ATP synthesis or hydrolysis [1–4].

In mitochondria the catalytic activity of the enzyme is regulated by a constituent subunit: the ATPase (EC 3.6.1.3) F_1 inhibitor protein, which is a low molecular weight, water-soluble and heat-stable protein [7,8]. Studies carried out in our laboratory have recently shown that F_1 inhibitor

protein can also modulate the proton conductivity in the H^+-ATPase complex in loosely coupled submitochondrial particles in the absence of ATP hydrolysis or synthesis [9,10].

In spite of the considerable progress made in the elucidation of the protein structure of H^+-ATPase [1–4] the mechanism of proton conduction by the membrane moiety (F_0) and of its coupling to the catalytic process in F_1 are still under debate [1–4]. Various authors have used chemical modification to identify critical aminoacid residues involved in the catalytic mechanism [11–18] and proton conduction [6,19–24]. In particular it has been reported that the histidine chemical modifier diethylpyrocarbonate inhibits the ATPase activity of purified F_1 [15] as well as H^+ conduction in purified F_0 from *Escherichia coli* [23].

In this paper a study of the effect of diethylpyrocarbonate on ATPase activity and passive H^+ conduction of the H^+-ATPase complex in submitochondrial particles is reported. The results obtained show that diethylpyrocarbonate treatment of sonic submitochondrial particles results in inhibition of ATPase activity and stimulation of oligomycin-sensitive H^+ conductivity.

The inhibition of ATPase activity is correlated with modification of histidine residues in F_1, the stimulation of passive H^+ conduction with modification of histidine residues in F_1 inhibitor protein. The modified inhibitor protein looses its capacity to inhibit ATP hydrolysis and proton conduction in the ATPase complex.

Materials and Methods

Oligomycin, *N,N'*-dicyclohexylcarbodiimide, valinomycin and diethylpyrocarbonate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); phospho*enol*pyruvate, pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27), β-nicotinamide adenine dinucleotide reduced form, adenosin-5-triphosphate and catalase from Boehringer Mannheim (Mannheim, F.R.G.). All other chemicals were of high purity grade.

Preparation of submitochondrial particles. Heavy bovine-heart mitochondria were prepared as described by Löw and Vallin [25], EDTA-submitochondrial particles were prepared, as described by Lee and Ernster [26], by exposure of bovine heart mitochondria to ultrasonic energy in the presence of EDTA at pH 8.5. The ATPase protein inhibitor was removed by passing EDTA-treated submitochondrial particles through a Sephadex column (Sephadex-EDTA-treated submitochondrial particles) [27]. F_1-deprived particles were obtained by sequential treatment of EDTA-treated submitochondrial particles with Sephadex chromatography and urea [27].

Purification of F_1 and F_1 inhibitor protein. Purification of F_1 was prformed by the chloroform extraction described by Beechey et al. [28]. This procedure is particularly convenient for being easy and rapid; it results, however, in a lower activity of the ATPase as compared to other preparations. F_1 inhibitor protein was prepared from Mg-ATP sonic submitochondrial particles [25] by the method of Kanner et al. [29] generally used for isolation of F_1 inhibitor protein from purified ATPase complex [30–32]. The polyacrylamide gel electrophoresis of the purified protein performed on slab gel of linear polyacrylamide gradient (12–20%) as described in Ref. 22, showed only one band of apparent M_r of 10500 (see Ref. 9).

Measurements of ATPase activity and H^+ conductivity. The ATPase activity was determined in the presence of added pyruvate kinase, phospho*enol*pyruvate and lactate dehydrogenase by following NADH oxidation spectrophotometrically as described in Ref. 33.

For measurements of proton conduction the relaxation of respiratory proton gradient was followed in sonic particles (3 mg protein/ml) incubated in a reaction mixture containing: 250 mM sucrose, 30 mM KCl, 0.5 μg valinomycin/mg particle protein, 0.2 mg/ml purified catalase and 20 mM succinate as respiratory substrate, final volume 1.5 ml (pH 7.5). Incubation was carried out in a glass vessel, under a constant stream of N_2, at 25 °C. Respiration-driven proton translocation was activated by repetitive pulses of 1–3% H_2O_2 (5 μl/ml). The pH changes, monitored potentiometrically were converted into proton equivalents by double titration with standard HCl and KOH [33]. The anaerobic release of respiratory proton gradient was inhibited by 70–80% by the specific inhibitors: oligomycin and DCCD [22,24,33].

Protein concentration was determined by the Lowry method [34].

Treatment of sonic particles and F_1 inhibitor protein with diethylpyrocarbonate. For diethylpyrocarbonate treatment, the particles were incubated with the reagent, at the concentrations reported in the legend to figures and tables, for 10 min at room temperature in phosphate buffer at pH 7.2; then the reaction was stopped by 10-times dilution with the buffer and the suspension was centrifuged at $105\,000 \times g$ for 10 min. The pellet was washed 2 times with 0.25 M sucrose.

For F_1 inhibitor protein treatment with diethylpyrocarbonate, 5 mg of purified protein were incubated with the reagent for 10 min, at room temperature in phosphate buffer at pH 7.2; then the suspension was dialyzed for 24 h against 2 l of 0.25 M sucrose. The dialyzed protein was collected and incubated 20 min with the particles at the concentration reported in the legend to figures and tables.

Results

Effects of diethylpyrocarbonate treatment of submitochondrial particles on ATPase activity and proton conduction

Modification of EDTA-treated submitochondrial particles with diethylpyrocarbonate resulted in extensive inhibition of ATPase activity and dramatic increase of the rate of anaerobic release of respiratory proton gradient (Table I).

However, whilst the inhibition of ATPase activity persisted, the promotion of proton conduction disappeared when the ATPase inhibitor protein was removed from the particles (Sephadex-EDTA-treated submitochondrial particles).

F_1 was isolated from diethylpyrocarbonate-treated EDTA-treated submitochondrial particles. The electrophoretic pattern of this F_1 preparation was similar to that obtained from normal particles (not shown), but the ATPase activity was severely inhibited (Fig. 1). The optical spectrum of F_1 purified from diethylpyrocarbonate-treated EDTA-treated submitochondrial particles showed a maximum of absorbance at 240 nm which was partially abolished by hydroxylamine. This is characteristic for histidine modification by diethylpyrocarbonate [35].

It can be noted that in particles deprived of F_1 inhibitor protein (Sephadex-EDTA-treated submitochondrial particles) or F_1 (urea-treated submitochondrial particles) diethylpyrocarbonate did not stimulate the anaerobic release of respiratory proton gradient, but, instead, caused some inhibition (Table I). This is apparently due to modification of histidine residues in membrane-integral components of F_0 [23] covered in EDTA-treated submitochondrial particles by F_1 inhibitor protein and F_1.

TABLE I

EFFECT OF DIETHYLPYROCARBONATE TREATMENT OF SUBMITOCHONDRIAL PARTICLES WITH VARIOUS DEGREES OF RESOLUTION OF H^+-ATPase COMPLEX ON ATPase ACTIVITY AND ON ANAEROBIC RELEASE OF RESPIRATORY PROTON GRADIENT

For preparation of EDTA-treated submitochondrial particles, urea-treated submitochondrial particles, treatment of particles with diethylpyrocarbonate and proton translocation, see under Materials and Methods. For determination of ATPase activity, submitochondrial particles (50 μg/ml) were suspended in a reaction mixture containing: 200 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 10 units lactate dehydrogenase, 20 mM Tris-HCl, 0.1 mM NADH, 1 mM phospho*enol*pyruvate, 4 units pyruvate kinase (pH 7.4). After 2 min incubation the reaction started by addition of 0.1 mM ATP and the oxidation of NADH was followed at 340 nm.

Submitochondrial particles	ATPase activity (μmol ATP hydrolyzed per min per mg protein)		Anaerobic release of respiratory proton gradient $1/t_{1/2}$ (s^{-1})	
	control	+ diethylpyrocarbonate (1 mM)	control	+ diethylpyrocarbonate (1 mM)
EDTA-treated submitochondrial particles	1.20	0.22	1.00	6.67
Sephadex-EDTA-treated submitochondrial particles	2.88	0.38	2.00	1.25
urea-treated submitochondrial particles	–	–	2.74	2.04

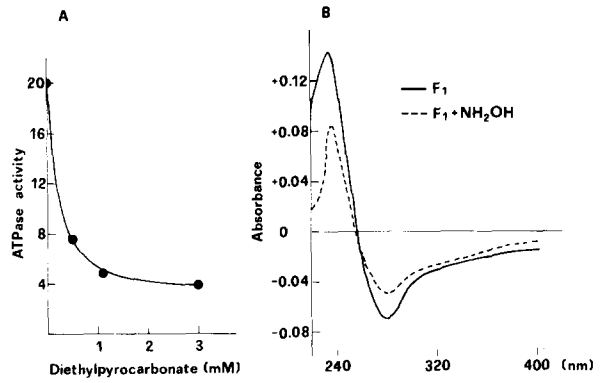

Fig. 1. Inhibition of ATPase activity of chloroform-extracted F_1 from diethylpyrocarbonate-treated EDTA-treated submitochondrial particles. For EDTA-treated submitochondrial particles preparation, diethylpyrocarbonate treatment and F_1 preparation, see under Materials and Methods. For ATPase activity determination (Fig. 1A), see legend to Table I with the difference that 5 μg of F_1/ml were used. The ATPase activity is expressed as μmol ATP hydrolyzed per min per mg protein. For the spectral analysis (Fig. 1B), 50 μg of F_1, extracted from 1 mM diethylpyrocarbonate-treated EDTA-treated submitochondrial particles, were suspended in 1 ml of phosphate buffer (pH 7.2) (solid line). The spectrum presented by dotted line refers to diethylpyrocarbonate-treated F_1 suspension to which 50 μl of 2 M NH_2OH were added.

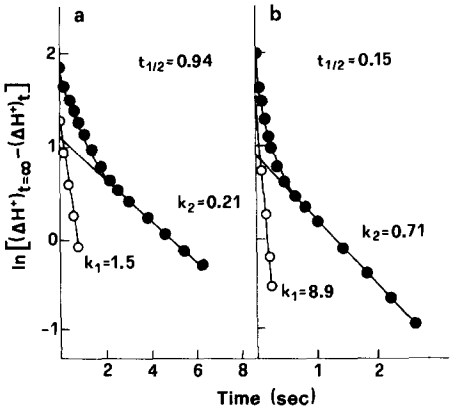

Fig. 2. Effect of diethylpyrocarbonate on kinetics of anaerobic release of respiratory proton gradient in EDTA-treated submitochondrial particles. For EDTA-treated submitochondrial particles preparation, diethylpyrocarbonate treatment and measurement of proton translocation, see under Materials and Methods. Mathematical analysis was carried out as described in Ref. 33. (a) EDTA-treated submitochondrial particles control; (b) EDTA-treated submitochondrial particles treated with 1 mM diethylpyrocarbonate.

Anaerobic release of respiratory proton gradient shows a biphasic pattern that can be resolved in two first-order processes (Fig. 2; see also Refs. 20–22 and 33); both are expressions of proton conduction by the ATPase complex as judged from their depression by oligomycin or DCCD [22,33] Diethylpyrocarbonate treatment of EDTA-treated submitochondrial particles stimulated both first-order phases, but the fast phase was more profoundly affected than the slow one (Fig. 2).

The titration curves reported in Fig. 3 show that the stimulation of anaerobic release of respiratory proton gradient increased with diethylpyrocarbonate concentration up to a concentration of 1 mM. At higher concentrations of diethylpyrocarbonate, the stimulatory effect decreased. The stimulation was abolished by oligomycin and DCCD (Fig. 3), indicating that the diethylpyrocarbonate effect is due to modification of the H^+-ATPase complex.

Fig. 4 shows that diethylpyrocarbonate treatment of EDTA-treated submitochondrial particles decreased the concentration of oligomycin causing 50% inhibition of proton conduction from 0.4 μg oligomycin/mg particle protein to 0.1 μg

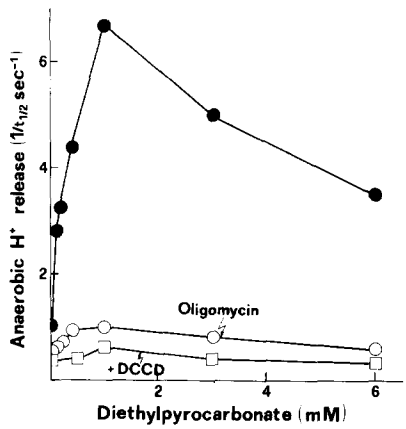

Fig. 3. Titration of the effect of diethylpyrocarbonate treatment of EDTA-treated submitochondrial particles on H^+-translocation induced by oxygen pulses of anaerobic succinate supplemented EDTA-treated submitochondrial particles. For experimental conditions, particles preparation and diethylpyrocarbonate treatment see under Materials and Methods and legend to Fig. 2. Symbols: ●——●, no addition; ○——○, +1 μg/mg prot oligomycin 10 min incubation; □——□, +50 μM DCCD 10 min incubation.

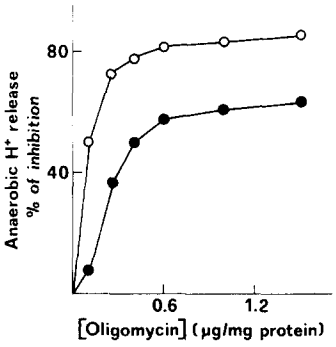

Fig. 4. Titration of oligomycin inhibition of anaerobic release of respiratory proton gradient. For EDTA-treated submitochondrial particles preparation, diethylpyrocarbonate treatment and experimental conditions, see under Materials and Methods and legend to Fig. 2. Symbols: ●———●, control; ○———○, EDTA-treated submitochondrial particles treated with 1 mM diethylpyrocarbonate. The numbers are percentages of the value of $1/t_{1/2}$ of anaerobic release of respiratory proton gradient that were: 1 s^{-1} for control EDTA-treated submitochondrial particles and 6.6 s^{-1} for diethylpyrocarbonate treated EDTA-treated submitochondrial particles.

oligomycin/mg particle protein. No difference in the titer of oligomycin was, on the other hand, observed in urea-treated submitochondrial particles prepared from EDTA-treated submitochondrial particles treated with diethylpyrocarbonate with respect to control urea-treated submitochondrial particles (not shown).

Effect of purified F_1 inhibitor protein on proton conduction by H^+-ATPase complex in sonic submitochondrial particles

As shown in Tables I and II removal of F_1 inhibitor protein from EDTA-treated submitochondrial particles by Sephadex chromatography resulted in (2–2.5)-fold increase of ATPase activity and proton conductivity. Both these stimulated activities were inhibited by the specific F_0 inhibitor oligomycin (not shown) [33]. The stimulation was completely reversed by the addition of inhibitor protein (Table II).

The small inhibitory effects exerted by F_1 inhibitor protein on ATP hydrolysis and proton conductivity in EDTA-treated submitochondrial particles may derive from partial loss or displace-

TABLE II

EFFECT OF F_1 INHIBITOR PROTEIN ON ATPase ACTIVITY AND ANAEROBIC RELEASE OF RESPIRATORY PROTON GRADIENT IN SUBMITOCHONDRIAL PARTICLES WITH VARIOUS DEGREES OF RESOLUTION OF H^+-ATPase COMPLEX

Preparations of submitochondrial particles, F_1 and F_1 inhibitor protein were carried out as described under Materials and Methods. Preincubation procedure: (a) For ATPase activity, submitochondrial particles (3–5 mg protein/ml) were preincubated, if indicated, with F_1 inhibitor protein (4 μg/mg particle protein) for 20 min at room temperature. Then 50 μg particle protein (or 5 μg purified F_1) were added to 1 ml of the mixture described in the legend to Table I and the ATPase activity was determined spectrophotometrically by following NADH oxidation at 340 nm. (b) For H^+ conduction submitochondrial particles (20 mg/ml) were preincubated with F_1 inhibitor protein as described above; then 0.23 ml of the suspension were added to 1.27 ml of the reaction mixture described under Materials and Methods and H^+ conductivity was determined by following potentiometrically anaerobic release of respiratory proton gradient as described under Materials and Methods. For reconstitution of oligomycin sensitive ATPase activity, EDTA-treated submitochondrial particles (15 mg protein/ml) were incubated with 5 mM $MgCl_2$ and 1.5 mg/ml of F_1 (or 1.5 mg/ml F_1 + 50 μg/ml F_1 inhibitor protein) for 20 min; then 50 μg of particle protein for ATPase activity determination (or 3 mg of particle protein for H^+ conductivity determination) were added to 1 ml (1.27 ml) of the reaction mixture.

	ATPase activity (μmol ATP hydrolized/per min per mg protein)		Anaerobic H^+ release $1/t_{1/2}$ (s^{-1})	
	control	+ F_1 inhibitor protein	control	+ F_1 inhibitor protein
EDTA-treated submitochondrial particles	1.25	0.92	1.00	0.75
Sephadex-EDTA-treated submitochondrial particles	2.88	0.90	2.00	0.91
Urea-treated submitochondrial particles	0.08	0.08	2.74	2.80
Urea-treated submitochondrial particles + F_1	1.00	0.50	1.67	1.11

ment of inhibitor protein in these particles.

Table II shows that F_1 inhibitor protein had no effect at all on proton conductivity in particles deprived of F_1 (urea-treated submitochondrial particles). F_1 inhibitor protein exerted, on the other hand, marked inhibitory effect on ATPase activity and proton conduction when the oligomycin-sensitive H^+-ATPase was reconstituted by addition of purified F_1 to urea-treated submitochondrial particles.

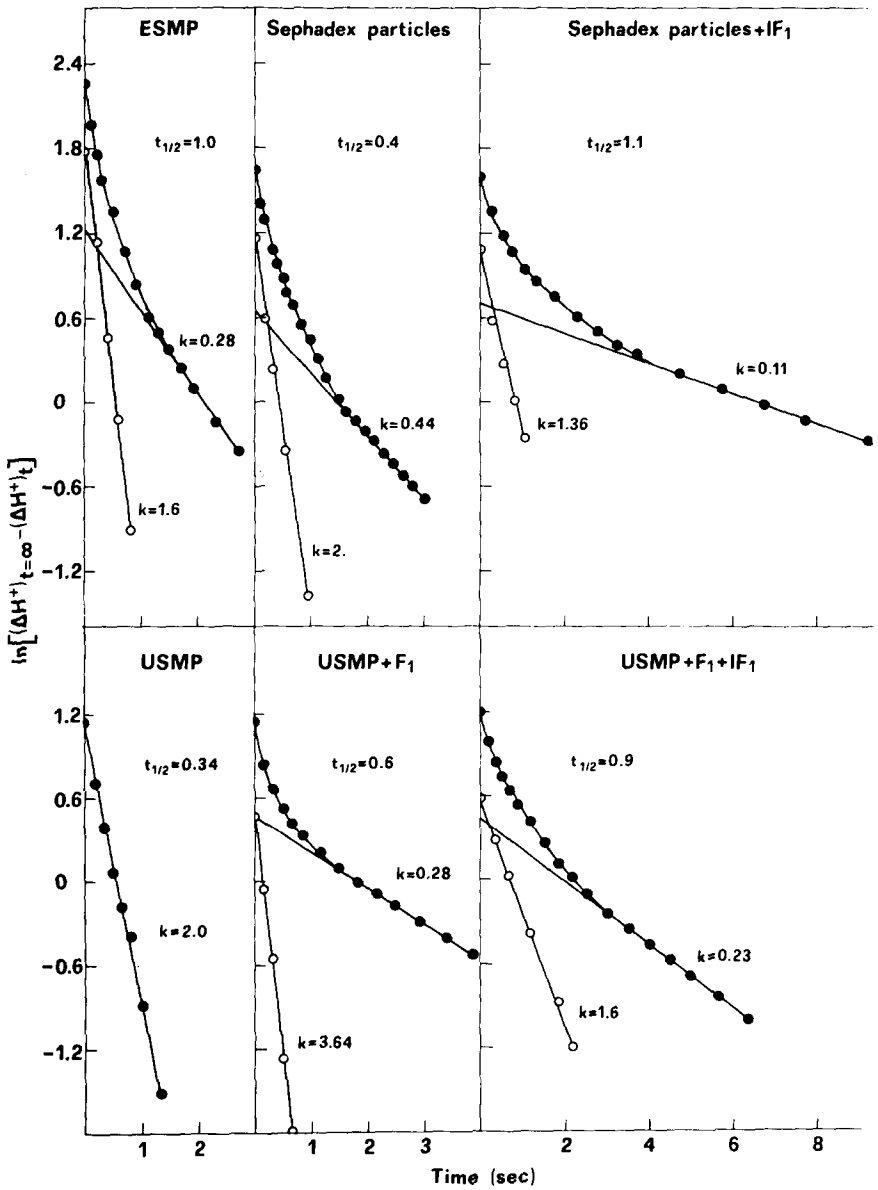

Fig. 5. Double exponential analysis of the kinetics of anaerobic release of respiratory proton gradient from sonic submitochondrial particles. Effect of resolution and reconstitution of H^+-ATPase complex. For particles, F_1 and F_1 inhibitor protein preparations, see under Materials and Methods. For resolution and reconstitution of the H^+-ATPase complex, see under Materials and Methods and legend to Table II. For kinetic analysis see legend to Fig. 2. ESMP, EDTA-treated submitochondrial particles; IF_1, F_1 inhibitor protein; USMP, urea-treated submitochondrial particles.

Fig. 5 shows that removal of F_1 inhibitor protein from EDTA-treated submitochondrial particles by Sephadex chromatography caused acceleration of both the first-order phases of anaerobic release of respiratory proton gradient (see also Ref. 33). Addition of inhibitor protein to Sephadex-EDTA-treated submitochondrial particles inhibited both phases.

Removal of F_1 by urea treatment of Sephadex-EDTA-treated submitochondrial particles changed the kinetic pattern of anaerobic H^+ release in a monophasic first-order process. Reconstitution of H^+-ATPase complex by addition of purified F_1 to depleted vesicles (urea-treated submitochondrial particles) caused an inhibition of the overall rate of proton back-flow and restoration of the biphasic pattern. Addition of purified F_1 inhibitor protein to this reconstituted system inhibited the overall rate of anaerobic release of respiratory proton gradient, being more effective on the fast phase of proton relaxation.

In the experiments of Fig. 6, samples of Sephadex-EDTA-treated submitochondrial particles were incubated with 4 μg F_1 inhibitor protein/mg particle protein in the presence of 5 mM $MgCl_2$ at pH's from 6.4 to 8.2. After 20 min incubation at room temperature small aliquots (50 μg particle protein for ATPase activity, and 3 mg particle protein for H^+ conduction) were added to the reaction mixtures for measurement of ATPase activity and proton conductivity.

The inhibitory action of F_1 inhibitor protein on ATPase activity was maximal at pH's below 7 and declined at alkaline pH's [7,36–39]. The inhibitory action exerted by inhibitor protein on H^+ conduction by H^+-ATPase complex exhibited a similar pH dependence (Fig. 6).

Fig. 7 shows the optical differential spectrum of purified F_1 inhibitor protein treated with 0.5 mM diethylpyrocarbonate. The spectrum showed a maximum of absorbance at 240 nm which was depressed by hydroxylamine. This is a characteristic for the formation of *N*-ethoxyformylhistidine [35]. The suppression of the 240 nm absorbance by hydroxylamine amounted, using an extinction coefficient for *N*-ethoxyformilhistidine of 3200 $M^{-1} \cdot cm^{-1}$ [35], to 0.84 mol histidine per mol F_1 inhibitor protein.

Modification of histidine residues by diethylpy-

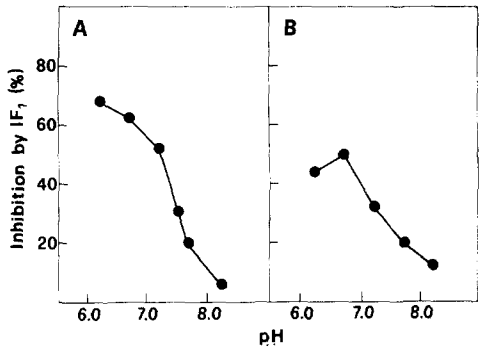

Fig. 6. pH dependence of the inhibition by F_1 inhibitor protein (IF_1) on ATPase activity and anaerobic release of respiratory proton gradient in sephadex-EDTA-treated submitochondrial particles. For preparation of Sephadex-EDTA-treated submitochondrial particles and of F_1 inhibitor protein, see under Materials and Methods. The assay of inhibition was performed a sdescribed in the text. The pH values indicated were those of the preincubation medium of particles with F_1 inhibitor protein. The enzyme assays were uniformly done at pH 7.4. The percentage inhibition was calculated from each set of experiments at the same pH in the absence and presence of F_1 inhibitor protein. The ATPase activity of Sephadex-EDTA-treated submitochondrial particles steadily decreased by about 12% form 8.2 to 7.4 and by about 37% from 7.4 to 6.2. The proton conductivity was constant from pH 8.2 to pH 7.2, decreased by about 20% from pH 7.2 to pH 6.7 and was practically constant ($1/t_{1/2} = 2$) until pH 6.2 (A) ATPase activity; (B) anaerobic release respiratory proton gradient.

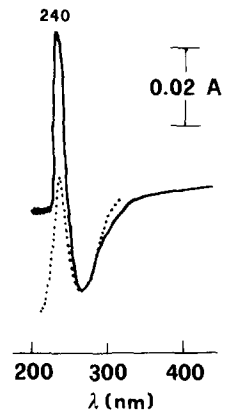

Fig. 7. Optical differential spectrum of purified F_1 inhibitor protein treated with diethylpyrocarbonate. For purification of F_1 inhibitor protein and its treatment with diethylpyrocarbonate, see under Materials and Methods. For the spectral analysis 145 μg F_1 inhibitor protein treated with diethylpyrocarbonate were suspended in 1 ml of P_i buffer pH 7.2 (solid line). The spectrum presented by dotted line refers to diethylpyrocarbonate treated inhibitor protein to which 50 μl of 2 M NH_2OH were added. The M_r of inhibitor protein was taken 10000 [41].

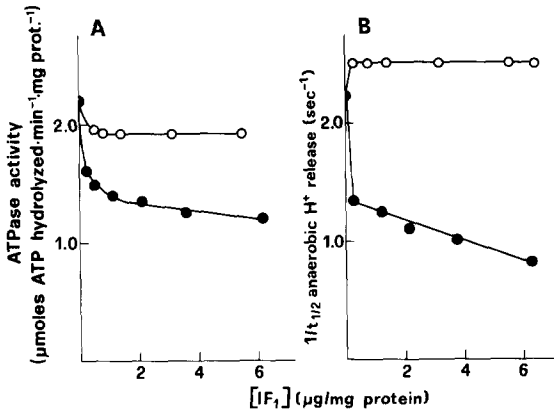

Fig. 8. Effect of diethylpyrocarbonate treatment of purified F_1 inhibitor protein on its inhibition of ATPase activity and anaerobic release of respiratory proton gradient in sephadex-EDTA-treated submitochondrial particles. Sephadex-EDTA-treated submitochondrial particles, F_1 inhibitor protein and its treatment with diethylpyrocarbonate were carried out as described under Materials and Methods. ATPase activity and rate of anaerobic release of respiratory proton gradient, were carried out as described under Materials and Methods and in the legend to Table I. (A) ATPase activity; (B) anaerobic release of proton gradient. Symbols: ●———●, F_1 inhibitor protein; ○———○, F_1 inhibitor protein modified by treatment with 0.5 mM diethylpyrocarbonate.

rocarbonate in purified F_1 inhibitor protein suppressed its activity as shown by the observation that addition of the modified inhibitor protein to Sephadex-ESMP had no effect on ATPase activity and on proton conductivity (Fig. 8). It should be recalled that bovine F_1 inhibitor protein has five histidine residues located in a cluster of external residues [40]. The present results show that modification of one out of the five histidines is enough to suppress inhibitor protein activity. This, however, does not necessarily mean that diethylpyrocarbonate modifies selectively one of the five histidine residues located in a specific position. Furthermore, it should be recalled that none of the histidine residues of F_1 inhibitor protein is conserved among species [41].

Discussion

The data reported in this paper show that treatment of sonic submitochondrial particles with diethylpyrocarbonate results in inhibition of ATPase activity and stimulation of oligomycin sensitive passive proton conduction by the ATPase complex.

The inhibition of the ATPase activity is clearly due to modification of the F_1 sector. In fact the inhibition of ATP hydrolysis persisted in the F_1 purified from diethylpyrocarbonate-treated EDTA-treated submitochondrial particles, which showed the absorbance spectrum characteristic for modified histidine.

In sonic submitochondrial particles the proton conductivity of the H^+-ATPase complex is modulated by the natural inhibitior protein of the complex (F_1 inhibitor protein) [9,10]. Removal of this protein from the particles results in stimulation of oligomycin-sensitive ATPase activity and proton conduction. Both these stimulated activities are inhibited by adding back purified inhibitor protein to the particles.

The stimulation of oligomycin-sensitive passive H^+ conduction by diethylpyrocarbonate treatment of EDTA-treated submitochondrial particles was lost after removal of F_1 inhibitor protein from the particles. Treatment of these particles with diethylpyrocarbonate caused inhibition of proton conduction resulting from modification of membrane integral F_0 components (see Ref. 23).

Removal of F_1 inhibitor protein from EDTA-treated submitochondrial particles increased the inhibitory action of oligomycin. This probably derives from enhanced accessibility of oligomycin to its site of inhibition [33]. Diethylpyrocarbonate treamtent of EDTA-treated submitochondrial particles also enhanced the inhibitory potency of oligomycin.

These observations indicate that diethylpyrocarbonate treatment of EDTA-treated submitochondrial particles results in modification of histidine residues essential for a correct functional interaction of F_1 inhibitor protein with the ATPase complex. This is directly verified by the finding that modification of histidine residues in the isolated inhibitor protein abolished the inhibitory activity that it exerts on ATP hydrolysis and proton conduction when added back to submitochondrial particles deprived of inhibitor protein.

F_1 inhibitor protein in addition to its regulatory role on the catalytic activity on the ATPase com-

plex appears to play a role in the control of the proton conductivity of the F_0-F_1 complex (see also Refs. 9 and 10). The present results (see also Ref. 32) indicate that the histidine residues in F_1 inhibitor protein are essential for the correct interaction of inhibitor protein with the complex and its functional activity.

It has been proposed that pH affects the conformation of F_1 inhibitor protein. This can apparently exist in two conformations [40], one predominant at pH 7.5 which is a non-binding form, the other, predominant at pH lower than 7, is a binding form with a helical region with high hydrophobicity [42], which probably interacts with F_1. The pH dependence of the inhibitory effects exerted by F_1 inhibitor protein on the ATPase activity and proton conduction may be related to this conformational transition. The pH profile of the activities of F_1 inhibitor protein shows a pK_a around 7.0. This would indicate that protonation of histidine represents a critical event in the pH-dependent conformational transition and functional activity of inhibitor protein.

Protonated histidine residues may contribute to the binding of F_1 inhibitor protein to other subunits in the complex through salt bridges with negatively charged residues. It is possible that binding of F_1 inhibitor protein to the β-subunit of F_1 [43] results in a conformational change which promotes binding of F_1 to F_0 with depression of proton conductance. Hashimoto et al. [44,45] have isolated from mitochondria two proteins which stabilize the formation of an F_0F_1-F_1 inhibitor protein complex. These proteins don's have any effect on the binding inhibitor protein to soluble F_1 [45].

F_1 inhibitor protein might mediate the binding of F_1 to these proteins of the membrane sector, thus acting as a component of the gate of the H^+-ATPase complex.

Acknowledgements

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