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Effect of Chemical Modifiers of Amino Acid Residues on Proton Conduction by the H^+-ATPase of Mitochondria

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Abstract

The effect of chemical modifiers of amino acid residues on the proton conductivity of H^+-ATPase in "inside out" submitochondrial particles has been studied. Treatment of submitochondrial particles prepared in the presence of EDTA (ESMP) with the arginine modifiers, phenylglyoxal or butanedione, or the tyrosine modifier, tetranitromethane, caused inhibition of the ATPase activity. Phenylglyoxal and tetranitromethane also caused inhibition of the anaerobic release of respiratory $\Delta\mu H^+$ in ESMP as well as in particles deprived of F_1 (USMP). Butanedione treatment caused, on the contrary, acceleration of anaerobic proton release in both particles. The inhibition of proton release caused by phenylglyoxal and tetranitromethane exhibited in USMP a sigmoidal titration curve. The same inhibitory pattern was observed with oligomycin and with *N,N'*-dicyclohexylcarbodiimide. In ESMP, relaxation of $\Delta\mu H^+$ exhibited two first-order phases, both an expression of the H^+ conductivity of the ATPase complex. The rapid phase results from transient enhancement of H^+ conduction caused by respiratory $\Delta\mu H^+$ itself. Oligomycin, *N,N'*-dicyclohexylcarbodiimide, and tetranitromethane inhibited both phases of H^+ release, and butanedione accelerated both. Phenylglyoxal inhibited principally the slow phase of H^+ conduction. In USMP, H^+ release followed simple first-order kinetics. Oligomycin depressed H^+ release, enhanced respiratory $\Delta\mu H^+$, and restored the biphasicity of H^+ release. Phenylglyoxal and tetranitromethane inhibited H^+ release in USMP without modifying its first-order kinetics. Butanedione treatment caused biphasicity of H^+ release from USMP, introducing a very rapid phase of H^+ release. Addition of soluble F_1 to USMP also restored biphasicity of H^+ release. A mechanism of proton conduction by F_o is discussed based on involvement of tyrosine or other hydroxyl residues, in series with the DCCD-reactive acid residue. There are apparently two functionally different species of arginine or other basic residues: those modified by phenylglyoxal, which facilitate H^+ conduction, and those modified by butanedione, which retard H^+ diffusion.

Key Words: H^+-ATPase; proton conduction; amino acid modification.

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Introduction

The H^+-ATPase of mitochondria and other coupling membranes consists of two functional multipeptide moieties: a peripheral sector, F_1, the ATPase proper, and a membrane-integral sector, F_o (Senior, 1973; Pedersen, 1975; Kagawa, 1978; Fillingame, 1980). It has been shown that F_o functions as a transmembrane proton translocator in native (Hinkle and Horstmann, 1971; Papa *et al.*, 1977; Pansini *et al.*, 1978) and artificial phospholipid membranes (Okamoto *et al.*, 1977; Nelson *et al.*, 1977; Criddle *et al.*, 1977; Celis, 1980; Sigrist-Nelson and Azzi, 1980; Negrin *et al.*, 1980). In the mitochondrial H^+-ATPase there are additional polypeptide subunits: the oligomycin-sensitivity-conferring factor, F_{c2} or F_6, which seems to be involved in the binding of F_1 to F_o, and an ATPase protein inhibitor (Senior, 1973; De Pierre and Ernster, 1977).

In the H^+-ATPase, normally arranged in the membrane, proton conduction by F_o is compulsorily coupled to a hydrodehydration reaction catalyzed by F_1. Following displacement or removal of F_1, as it occurs in certain vesicular preparations of the inner mitochondrial membrane, the proton conductivity of F_o results in passive diffusion of protons, which can be blocked by oligomycin or DCCD[2] (Hinkle and Horstmann, 1971; Papa *et al.*, 1977; Pansini *et al.*, 1978). Proton conduction with similar characteristics is obtained when F_o is incorporated in artificial phospholipid membranes.

Considerable progress has been made in the elucidation of the protein structure of F_o (Kagawa, 1978; Fillingame, 1980; Sebald *et al.*, 1979b; Altendorf *et al.*, 1979), this providing an important basis for the elucidation of the molecular mechanism of proton conduction by F_o.

Previous work from this laboratory showed that anaerobic relaxation of the electrochemical proton gradient, $\Delta\mu H^+$, set up by respiration in submitochondrial particles, takes place practically through the proton-conducting pathway of the H^+ATPase (Papa *et al.*, 1977; Pansini *et al.*, 1978). Kinetic analysis of this process and studies with F_o- and F_1-inhibitors provided evidence that proton conduction in the H^+-ATPase is regulated by transmembrane $\Delta\mu H^+$ and interactions between charged groups in F_1 and F_o (Pansini *et al.*, 1978; Pansini *et al.*, 1979). In an attempt to identify the chemical nature of these groups we have examined the effect of arginine and tyrosine modifiers on ATPase activity and proton conduction in "inside out" vesicles of the inner mitochondrial membrane. It might be mentioned that two different arginine residues appear to be respectively involved in the ATPase activity and in the ATP-Pi exchange of the H^+-ATPase complex (Marcus *et al.*, 1976; Frigeri *et al.*, 1977). More recently it has been reported that

[2]DCCD is *N,N'*-dicyclohexylcarbodiimide.

modification with phenylglyoxal of arginine residues and of tyrosine with tetranitromethane depresses the proton conductivity of F_o from the thermophilic bacteria PS3 in liposomes (Sone *et al.*, 1979a; Sone *et al.*, 1981). The use of DCCD, a specific reagent for glutamic or aspartic acid, has suggested the role in proton translocation of a glutamic or aspartic residue of the 7,000–8,000-dalton proteolipid component of F_o (Sebald *et al.*, 1979b; Altendorf *et al.*, 1979).

The results of the present study provide evidence that arginine and tyrosine residues of the membrane sector play a specific role in proton conduction by H^+-ATPase of mitochondria. A molecular mechanism of proton conduction by F_o, based on involvement of these and other polar residues, is discussed.

Materials and Methods

Chemicals

Oligomycin, valinomycin, and *N,N'*-dicyclohexylcarbodiimide were obtained from Sigma Chemical Co. (St. Louis, Missouri); phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, β-nicotinamide adenine dinucleotide reduced form, adenosine 5-triphosphate, and catalase, from Boehringer (Mannheim, FRG); 2,3-butanedione, phenylglyoxal monohydrate, and tetranitromethane, from Aldrich Europe (Beerse, Belgium). All the other chemicals were of high purity grade.

Preparation of Submitochondrial Particles and Soluble F_1

"Inside out" submitochondrial particles were obtained by exposure of beef-heart mitochondria (Löw and Vallin, 1963) to ultrasonic energy in the presence of EDTA at pH 8.5 (ESMP) (Lee and Ernster, 1968; Pansini *et al.*, 1978). The inverted orientation of the inner mitochondrial membrane in submitochondrial particles was determined from cytochrome *c* stimulation of succinate and NADH respiration using the relation % inversion = (nonstimulated respiratory rate/stimulated rate) × 100, which is based on inaccessibility of cytochrome *c* to its reaction site in "inside out" vesicles (Harmon *et al.*, 1974). With the addition of an excess of cytochrome *c* (80 μM) to submitochondrial particles respiring with 10 mM succinate or 1 mM NADH, the % inversion was found to range from preparation to preparation from 96% to 100% (cf. Harmon *et al.*, 1974; Huang *et al.*, 1973). Sequential treatment of ESMP with Sephadex chromatography and urea (Racker and Horstmann, 1967) produced particles devoid of F_1 (USMP) as judged from the absence of any detectable ATPase activity.

Soluble ATPase (F_1) was prepared from beef-heart mitochondria as described by Horstmann and Racker (1970). For reconstitution USMP were incubated for 20 min at room temperature in a medium containing 130 μg protein F_1 per milligram protein USMP, 120 mM potassium phosphate buffer, pH 7.4, 5 mM $MgSO_4$, and 5 mM ATP (Racker and Horstmann 1967). After incubation the reconstituted particles were centrifuged at 105.000 *g* for 15 min and the pellet suspended in 0.25 M sucrose. The ATPase activity of F_1-reconstituted particles was 1.7 μmol ATP hydrolyzed per minute per milligram protein USMP. This ATPase activity was oligomycin sensitive.

Treatment of Submitochondrial Particles with Amino Acid Modifiers

2,3-Butanedione (1 M) was freshly prepared for each experiment in 0.1 M boric acid–borate buffer, pH 8, containing 0.1 mM EDTA (Frigeri *et al.*, 1977). Phenylglyoxal (0.25 M) was dissolved in 0.3 M borate buffer (Werber *et al.*, 1975). Submitochondrial particles, suspended in a basic reaction medium at pH 7.5 (Pansini *et al.*, 1978) (see legend to Fig. 2), supplemented with 20 mM succinate, were incubated for 10 min under a constant stream of N_2 in a glass vessel thermostated at 25° ± 0.01°C. Butanedione or phenylglyoxal were added and the incubation of the particles was continued for 20 min, the pH of the medium being adjusted to 7.5 with diluted HCl (Riordan, 1973).

Tetranitromethane, diluted with ethanol 1:10 (v/v) to give a concentration of 0.84 M, was added, at the final concentration given in the legends to the figures, to submitochondrial particles suspended in the basic reaction mixture at pH 7.5 supplemented with 20 mM succinate. The treatment was carried out under N_2 at room temperature for 30 min, after which the suspension was diluted with 7 volumes of cold 0.25 M sucrose and the particles spun down at 105,000 *g* for 10 min. The pellet was resuspended in sucrose and the particles, sedimented by centrifugation at 105.000 *g*, were

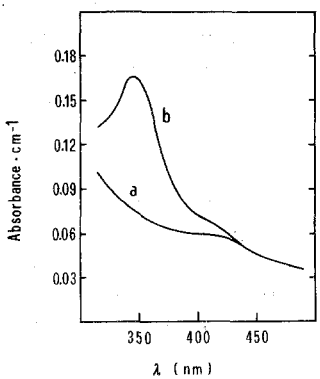

Fig. 1. Spectra of ESMP treated with tetranitromethane. ESMP (3 mg protein/ml) were treated with 0.21 μmol of tetranitromethane per milligram protein as described in the text in the reaction mixture described in the legend to Fig. 2. The absorbance was measured on a sample containing 0.2 mg protein/ml in 100 mM potassium phosphate buffer at pH 7.5 (a) Untreated ESMP; (b) ESMP treated with tetranitromethane.

collected in 0.25 M sucrose. The nitration of tyrosine residues in membrane proteins was verified by the appearance of tetranitromethane-treated particles (Fig. 1) of the absorbance peak at 350–360 nm characteristic for nitrotyrosyl (Riordan and Vallee, 1972).

DCCD as ethanolic solution was added at the concentrations indicated in the figures to submitochondrial particles suspended in the reaction mixture, and the measurements were carried out after 10 min preincubation.

Measurement of Proton Translocation

Submitochondrial particles, 3 mg protein/ml, were incubated in a reaction mixture containing 250 mM sucrose, 30 mM KCl, 0.5 μg valinomycin per milligram 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, thermostated at 25 ± 0.01°C by a glass jacket connected to a water bath. Respiration-driven proton translocation was activated by repetitive pulses of 1–3% H_2O_2 (5 μl/ml). The pH of the suspension was monitored potentiometrically with a Beckman combination electrode (No. 39030, Beckman Instruments International Geneva, Switzerland), connected to a Keithley differential electrometer amplifier (model 604, Keithley Instruments) and from this to a strip chart recorder (Leeds and Northrup). The overall response time of the pH recording system used was about 300 ms at 25°C (Papa *et al.*, 1979).

For the kinetic analysis of the anaerobic proton release from submitochondrial particles, the potentiometric traces were converted into proton equivalents by double titration with standard HCl and KOH and treated by a double exponential equation (Papa *et al.*, 1973).

Determination of ATPase Activity

The ATPase activity was determined in the presence of added pyruvate kinase, phosphoenolpyruvate and lactate dehydrogenase by following spectrophotometrically NADH oxidation at 340 nm.

The reaction mixture contained 250 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4, 0.1 mM NADH, 0.5 μg rotenone, 1 mM phosphoenolpyruvate, 0.1 mM ATP, 5 units lactate dehydrogenase, 2 units pyruvate kinase, and 20–30 μg protein submitochondrial particles in a final volume of 1 ml.

Results

Both the arginine modifiers, phenylglyoxal and butanedione, and the tyrosine modifier, tetranitromethane, produced inhibition of the ATPase

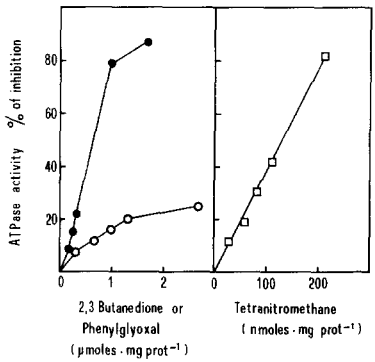

Fig. 2. Effect of arginine and tyrosine modifiers on ATPase activity. ESMP (3 mg protein/ml) were treated with 2,3-butanedione or phenylglyoxal as described under Materials and Methods in a reaction medium containing 200 mM sucrose, 30 mM KCl, 0.2 mg/ml catalase, and valinomycin (0.5 μg/mg protein). Tetranitromethane treatment was carried out as described under Materials and Methods. The ATPase activity was measured on a sample of treated ESMP (40 μg protein) diluted to 1 ml with a reaction mixture containing 200 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-HCl pH 7.4, 0.1 mM NADH, 0.5 μg rotenone, 1 mM phosphoenolpyruvate, 5 units lactate dehydrogenase, and 2 units pyruvate kinase. After 2 min of incubation the reaction was started by the addition of 0.1 mM ATP and the ATPase activity determined by following spectrophotometrically NADH oxidation at 340 nm. Symbols: ○, phenylglyoxal; ●, 2,3-butanedione. □, tetranitromethane. The values reported represent the percentage of inhibition of the rate of ATPase activity. Control value: 1 μmol ATP hydrolyzed per minute per milligram protein.

activity of ESMP. The titration curves presented in Fig. 2 show that, under the conditions used and in the presence of borate buffer, butanedione caused a much greater inhibition of the ATPase activity than phenylglyoxal. While the first modifier produced 50% inhibition at a concentration of 0.63 μmol/mg protein ESMP and almost complete inhibition at 1.7 μmol/mg protein, the latter caused only 25% inhibition at 2.7 μmol/mg protein. Tetranitromethane treatment was even more effective than butanedione; it caused, in fact, 50% inhibition at 130 nmol/mg protein and almost full inhibition at 210 nmol/mg protein.

Figure 3 shows the effect of treatment with amino acid modifiers on proton translocation elicited by oxygen pulses of anaerobic ESMP supplemented with succinate as respiratory substrate (Papa *et al.*, 1973). Phenylglyoxal and tetranitromethane caused a marked depression of the rate of anaerobic release of protons taken up by ESMP during the respiratory pulse, as indicated by the large increase of the $t_{1/2}$ of the process. It can, however, be noted that that while the inhibition of proton back flow by oligomycin (Fig. 3) or DCCD (Hinkle and Horstmann, 1971; Pansini *et al.*, 1978) resulted, as expected, in a significant enhancement of the extent of proton uptake at the aerobic steady state, the inhibitory effect exerted by phenylglyoxal and tetranitromethane treatment was, on the contrary, accompanied by depres-

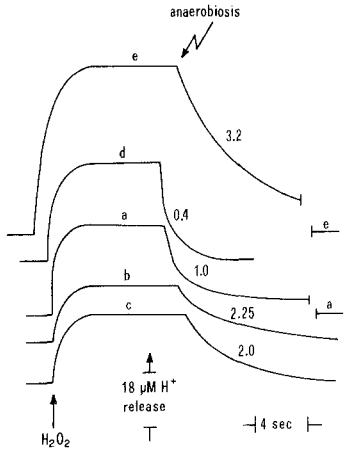

Fig. 3. Effect of oligomycin and amino acid modifiers on proton translocation induced by respiratory pulses in ESMP. ESMP (3 mg protein/ml), treated with amino acid modifiers as described in Fig. 2 were incubated in the reaction medium described under Materials and Methods. (a) Control; (b) ESMP treated with 2.5 μmol phenylglyoxal per milligram protein; (c) ESMP treated with 0.21 μmol tetranitromethane per milligram protein; (d) ESMP treated with 10 μmol 2,3-butanedione per milligram protein; (e) ESMP supplemented with 0.6 μg oligomycin per milligram protein. The numbers reported on the pH traces refer to the $t_{1/2}$ of proton release in anaerobiosis. It should be noted that duration of the cycles depends, in addition to the respiratory activity, on the amount of H_2O_2, added which could vary slightly from experiment to experiment. Direct controls showed that while 2,3-butanedione stimulated the rate of respiration, the other reagents depressed it.

sion of the initial rate and the extent of aerobic proton uptake by ESMP. This indicates that the treatment with these two reagents, besides inhibiting passive proton back flow, caused also depression of active proton uptake by ESMP supported by succinate respiration.

Butanedione treatment produced, in contrast to what was observed with phenylglyoxal and tetranitromethane, a marked stimulation of the anaerobic proton release from ESMP, without having a significant effect on the extent of respiratory $\Delta\mu H^+$ (Fig. 3).

In Fig. 4 the titration curves are presented for the inhibitory effects exerted by oligomycin and amino acid modifiers on the anaerobic relaxation of respiratory $\Delta\mu H^+$ in ESMP. It can be seen, from the relative enhancement of the $t_{1/2}$ of $\Delta\mu H^+$ relaxation caused by them, that oligomycin, DCCD, tetranitromethane, and phenylglyoxal were all almost equally effective in inhibiting H^+ conductivity. Treatment with each of these reagents caused a three- to five-fold enhancement of the $t_{1/2}$ of H^+ release.

Treatment of ESMP with butanedione caused, on the other hand, a pronounced acceleration of H^+ release, the $t_{1/2}$ of this process being lowered from 1 sec to 0.1 sec at 13.3 μmol/mg protein of butanedione. It should be mentioned that separate controls showed that butanedione, with or without borate buffer, had no effect whatsoever on H^+ conductivity in liposomes.

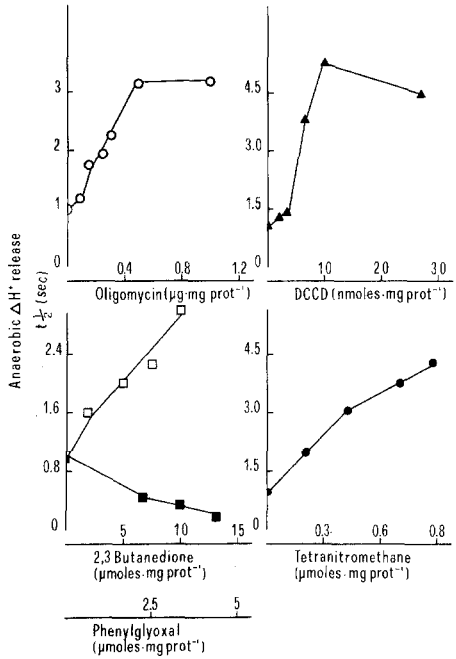

Fig. 4. Titration curves of the effect of oligomycin and amino acid modifiers on $t_{1/2}$ of passive proton release from ESMP. For experimental procedure and treatment with amino acid modifiers, see Materials and Methods and the legends to Figs. 2 and 3. Control value $t_{1/2}$ = 1 sec. Symbols: ○, oligomycin; ▲, DCCD; ■, 2,3-butanedione; □, phenylglyoxal; ●, tetranitromethane.

Table I. Effect of Arginine Modifiers on $t_{1/2}$ of Passive Proton Release from ESMP[a]

First treatment	Second treatment	$t_{1/2}$ anaerobic H^+ release (sec)
μmol/mg protein		
Control		1.2
Phenylglyoxal (2.5)		2.7
Phenylglyoxal (2.5);	2,3-Butanedione (10)	1.2
2,3-Butanedione (10)		0.5
2,3-Butanedione (10);	Phenylglyoxal (2.5)	1.1

[a]For experimental procedure and treatment with amino acid modifiers, see Materials and Methods and legend to Fig. 4

It can be noted that the titers for the effects of phenylglyoxal and butanedione on proton translocation were quite different from those observed for inhibition of the ATPase activity. This might be due to modification by the reagents of additional residues in the ATPase complex besides the arginine of F_1 involved in the ATPase activity (Marcus *et al.*, 1976; Frigeri *et al.*, 1977). The difference in the effects of phenylglyoxal and butanedione on proton translocation is due to modification by these two reagents of different residues in the ATPase complex. This is documented by the experimental data presented in Table I. These show that treatment with butanedione of ESMP, whose H^+ conductivity had been depressed by phenylglyoxal, still caused stimulation.

Similarly, phenylglyoxal added to ESMP, whose H^+ conduction had been promoted by butanedione, inhibited the process.

Removal of F_1 from ESMP allows the H^+ conductivity of F_o to freely manifest itself (Hinkle and Horstmann, 1971; Pansini *et al.*, 1978). In USMP the extent of inhibition of H^+ conduction by oligomycin was higher than in ESMP (Fig. 5). However, in USMP the titration curve of inhibition of H^+

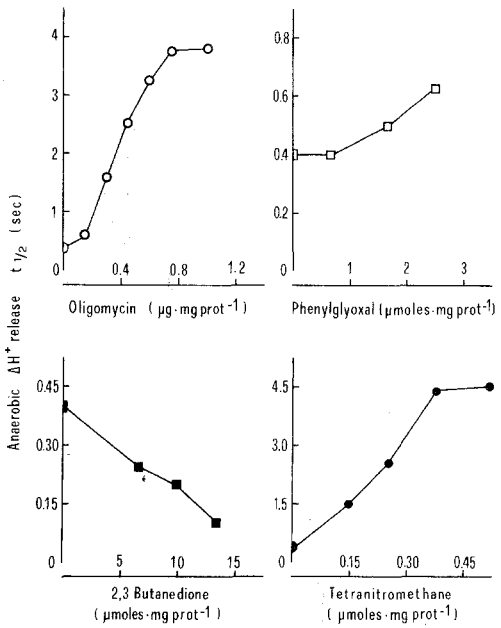

Fig. 5. Titration curves of the effect of oligomycin and amino acid modifiers on $t_{1/2}$ of passive proton release from USMP. For experimental procedure and treatment with amino acid modifiers, see Materials and Methods and the legends to Figs. 2 and 3. Control value $t_{1/2} = 0.4$ sec.

conduction by oligomycin showed a marked sigmoidicity, which was barely detectable in ESMP (see also Pansini *et al.*, 1978).

Treatment with amino acid modifiers caused inhibition of anaerobic release of respiratory $\Delta\mu H^+$ also in USMP. The inhibition of H^+ conduction caused by tetranitromethane treatment of USMP was, as in the case of

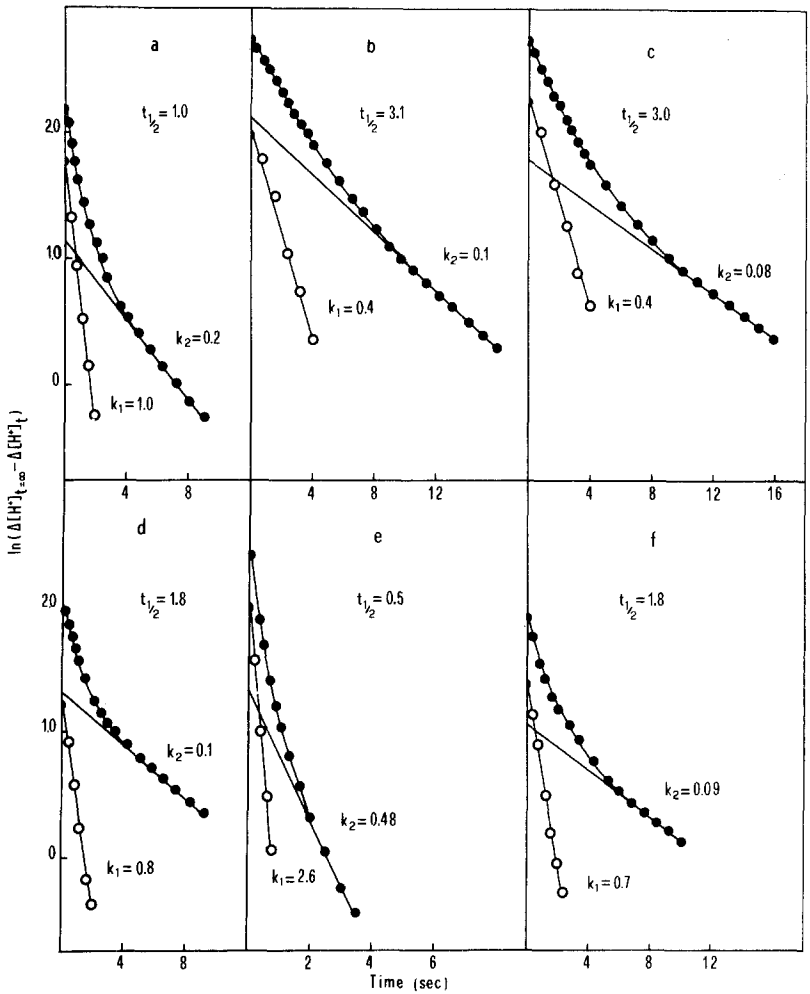

Fig. 6. Mathematical analysis of the effect of oligomycin and of amino acid modifiers on anaerobic proton release from ESMP. The kinetics of anaerobic proton release was mathematically resolved into two first-order processes as described by Papa *et al.* (1973). For experimental conditions see the legend to Fig. 3. (a) Control; (b) plus oligomycin (0.5 μg/mg protein); (c) plus DCCD (7 nmol/mg protein); (d) ESMP treated with phenylglyoxal (2.5 μmol/mg protein); (e) ESMP treated with 2,3-butanedione (10 μmol/mg protein); (f) ESMP treated with tetranitromethane (0.21 μmol/mg protein).

oligomycin, more extensive than in ESMP and exhibited a sigmoidal titration curve. On the other hand, the inhibition of H^+ conduction effected by phenylglyoxal treatment and the stimulation by butanedione treatment were less pronounced in USMP than in ESMP.

In ESMP the anaerobic relaxation of respiratory $\Delta\mu H^+$ can be mathematically resolved into a rapid and slow first-order phase. Both phases are an expression of the H^+ conductivity of H^+-ATPase as judged from the titer of inhibition by oligomycin and DCCD (Pansini *et al.*, 1978; Pansini *et al.*, 1979) (Fig. 6).

Figure 6 shows that tetranitromethane treatment caused, like oligomycin and DCCD, depression of both phases of H^+ release. Depression of H^+ release caused by phenylglyoxal treatment of ESMP was, on the other hand, principally due to inhibition of the slow phase of H^+ release.

Butanedione produced practically the same stimulation of both the rapid and slow phase of H^+ release.

In the particles devoid of F_1 (USMP) the relaxation of respiratory $\Delta\mu H^+$ did not exhibit the biphasic pattern characteristic of ESMP; H^+ release followed in this case simple first-order kinetics. Inhibition by oligomycin of H^+ release resulted in enhancement of respiratory $\Delta\mu H^+$ in USMP, up to the values observed in ESMP (Fig. 7). This was accompanied by reappearance of the biphasic pattern of H^+ release. The kinetic constants of the two phases of H^+ release were, however, depressed with respect to the constant of the single first-order process observed in untreated USMP (Fig. 7). The inhibition of H^+ release by tetranitromethane or phenylglyoxal treatment of USMP did not enhance, as already observed in ESMP, the extent of respiratory $\Delta\mu H^+$. These reagents depressed the anaerobic H^+ release from USMP without modifying its apparent first-order kinetics.

The stimulation of H^+ conduction caused by butanedione treatment exhibited a peculiar pattern. Butanedione restored the biphasic pattern of H^+ release, i.e., it resulted in a very rapid initial phase of proton release followed by a phase of restricted H^+ diffusion. This kinetic pattern induced by butanedione treatment resembles, under certain aspects, that observed upon reconstitution of USMP with soluble F_1. In fact, adding back F_1 to USMP restored the biphasic pattern of H^+ release by introducing an initial rapid phase, whose kinetic constant was significantly higher than the first-order kinetic constant of untreated USMP. However, this appearance of a rapid phase of H^+ release induced by F_1 was accompanied by a marked depression of the slow phase, which resulted in an overall increase of the $t_{1/2}$ of H^+ release (Fig. 7).

Figure 8 shows that, after treatment of USMP with a concentration of tetranitromethane which caused partial inhibition of anaerobic relaxation of respiratory $\Delta\mu H^+$, oligomycin caused no further inhibition of the process. It

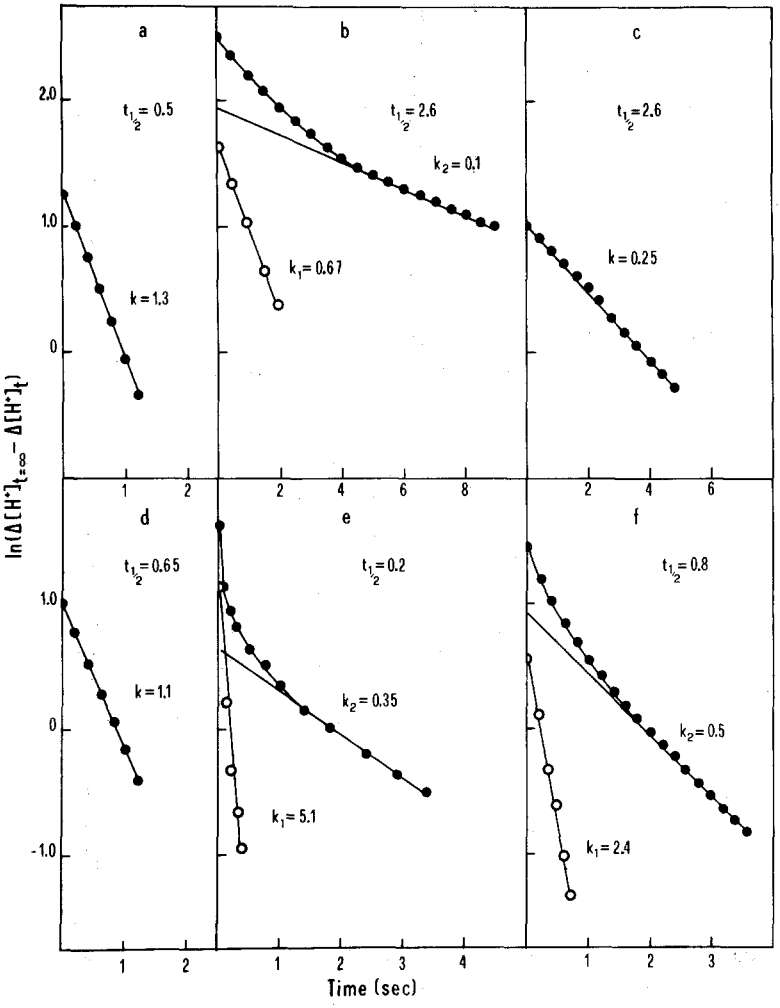

Fig. 7. Mathematical analysis of the effect of oligomycin, amino acid modifiers, and F_1 on anaerobic H^+ release from USMP. The kinetics of anaerobic proton release was mathematically resolved into two first-order processes as described by Papa *et al.* (1973). For experimental conditions see the legend to Fig. 3. (a) Control; (b) plus oligomycin (0.5 μg/mg protein); (c) USMP treated with tetranitromethane (0.21 μmol/mg protein); (d) USMP treated with phenylglyoxal (2.5 μmol/mg protein); (e) USMP treated with 2,3-butanedione (10 μmol/mg protein); (f) USMP reconstituted with purified F_1 (see Materials and Methods).

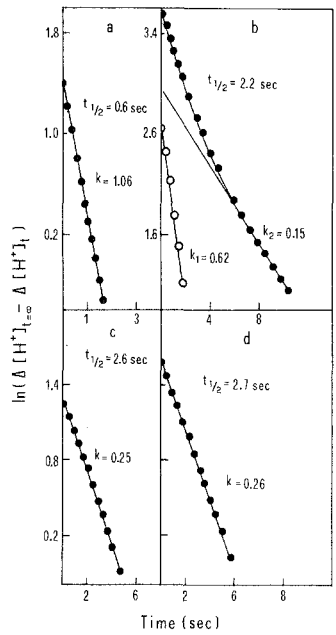

Fig. 8. Effect of tetranitromethane and oligomycin on anaerobic H^+ release from USMP. The kinetics of anaerobic proton release was analyzed mathematically as described by Papa *et al.* (1973). For experimental conditions see the legend to Fig. 3. (a) Control; (b) plus 0.5 oligomycin (μg/mg protein); (c) USMP treated with tetranitromethane (0.21 μmol/mg protein); (d) USMP treated with tetranitromethane (0.21 μmol/mg protein) were supplemented with oligomycin (0.5 μg/mg protein).

can be noted, on the other hand, that oligomycin, added to USMP treated with a concentration of phenylglyoxal causing partial inhibition of H^+ release, produced further inhibition (not shown).

Discussion

In "inside out" submitochondrial particles prepared in the presence of EDTA (ESMP) or devoid of F_1 (USMP) the anaerobic release of protons, taken up by the particles during respiration, is practically mediated by the F_o moiety of the H^+-ATPase. This is clearly documented by the titer of inhibition of H^+ release by oligomycin and DCCD (see also Pansini *et al.*, 1978), as well as by the specific effects exerted on the process by removal of F_1 and its recombination with F_1-deprived particles (see also Hinkle and Horstmann, 1971; Pansini *et al.*, 1979).

F_o isolated from mitochondria (Shchipakin *et al.*, 1976; Glaser *et al.*, 1980) or bacteria (Okamoto *et al.*, 1977; Negrin *et al.*, 1980) functions, when inserted in liposomes, as a transmembrane H^+ translocator which is inhibited by DCCD and also by oligomycin in the case of the mitochondrial material. Proton conductivity is also induced by incorporation in liposomes of the DCCD-binding proteolipid of F_o (Nelson *et al.*, 1977; Criddle *et al.*, 1977;

Celis, 1980; Sigrist-Nelson and Azzi, 1980), which can be considered as representing by its own the proton channel of H^+-ATPase.

Chemical modification with DCCD has enabled us to identify the role for proton conduction by F_o of the glutamic acid residue in position 65 (according to the numbering of *Neurospora Crassa* sequence) of the DCCD-binding proteolipid (Sebald *et al.*, 1979a; Sebald et al., 1979b). Tyrosine nitration with tetranitromethane or arginine modification with phenylglyoxal in F_o of thermophilic bacterium PS3 cause inhibition of H^+ conduction (Sone *et al.*, 1979a; Sone *et al.*, 1981). It seems that the critical residues modified belong to the DCCD-binding proteolipid component of PS3-F_o (Sone *et al.*, 1979b).

The effects produced by treatment of submitochondrial particles with arginine or tyrosine modifiers on anaerobic relaxation of respiratory $\Delta\mu H^+$ are retained when F_1 is removed from the particles. Thus the inhibition of proton release caused by phenylglyoxal and tetranitromethane and the stimulation caused by butanedione result from modification of amino acid residues of membrane integral polypeptide subunits.

It has been reported that genetic substitution of residues in the hydrophobic segment of the DCCD-binding proteolipid, where the critical acidic residue-65 is located, results in resistance to oligomycin (Sebald *et al.*, 1979b). The present observation that tetranitromethane treatment of USMP prevented further inhibition of H^+ conduction by oligomycin would indicate that the treatment resulted in nitration of the tyrosine residue in position 56. Another tyrosine is, however, present at position 42 (Sebald *et al.*, 1979b).

The position of the residues modified by phenylglyoxal in the mitochondrial as well as in the F_o sectors of H^+-ATPase of other organelles remains to be identified. It seems significant, however, that only one arginine is present in the DCCD-binding proteolipid of mitochondria at position 45 and this is also occupied by arginine in the other DCCD-binding proteolipid so far analyzed (Sebald et al., 1979b).

It may be surprising that treatment of submitochondrial particles with butanedione, which is even more specific than phenylglyoxal for arginine (Riordan, 1973; Borders and Zurcher, 1979), caused stimulation of H^+ release, instead of inhibition as found with phenylglyoxal. The experiments of Table I show that the two reagents react, in fact, with different residues. This can be due to specificity of attack of arginines of different subunits of F_o with differential accessibility to the two reagents or attack by one of the two reagents of residue(s) other than arginine.

There is evidence that the DCCD-binding proteolipid exists in the membrane as a hexamer (Sebald *et al.*, 1979a,b). The six monomers appear to function in a concerted way: modification by DCCD of 1/6 to 1/3 of the

proteolipid subunits results in inhibition of H^+ conduction (Sebald *et al.*, 1979a).

The observation that the titration curve for inhibition of H^+ conduction in submitochondrial particles by tetranitromethane or phenylglyoxal is sigmoidal, like that for the inhibition by oligomycin and DCCD (see Fig. 5), provides further evidence that the inhibition results in all the cases from modification of residues in the DCCD-binding proteolipid.

Each monomer of the DCCD-binding proteolipid has two hydrophobic segments of 20–25 residues (Sebald *et al.*, 1979b). These could span the membrane along an axis perpendicular to its plane. The critical residue-65 is in the middle of the carboxyl-terminal hydrophobic segment. The two hydrophobic segments have in addition a number of hydroxyl residues varying from species to species from three to seven (Sebald *et al.*, 1979b). We have recently discussed possible molecular mechanisms of H^+ conduction by F_o and have proposed (Papa and Guerrieri, 1981) that hydroxyl residues from adjoining chains, joined by hydrogen bonds (Nagle and Morowitz, 1978; Dunker and Marvin, 1978), can constitute a network, with the acidic residue-65 in the middle, along which protons can rapidly move across the membrane. The arginine-45 and other basic residues in the central loop or in the N-terminus of the DCCD-binding proteolipid could be involved in the access of H^+ at the entry mouth and their release from the exit mouth of the channel. Modification by phenylglyoxal of the basic residue at the entry mouth will depress proton conductivity, and modification by butanedione of the basic residue at the exit mouth will enhance proton conductivity.

The basic residues at the mouth of the F_o channel can be engaged in salt-bridges with acidic residues of the connection polypeptides of F_1 (Pansini *et al.*, 1979). Formation of salt-bridges can shift the pK of the residues engaged.

The generation of high transmembrane $\Delta\mu H^+$, more acidic in the internal phase, appears to induce high conductivity by H^+-ATPase in submitochondrial particles (Fig. 7; see also Pansini *et al.*, 1978; Pansini *et al.*, 1979). The same seems to be the case in chloroplasts (Portis *et al.*, 1975; Olivier and Jagendorf, 1976). In liposomes H^+ conductivity of F_o of the thermophilic bacterium PS3 (Okamoto *et al.*,) 1977; Sone *et al.*, 1981) and of chloroplasts (Sigrist-Nelson and Azzi, 1980) is enhanced at pH below 7, with a pH dependence which indicates involvement of a monoprotic site with a pK of 6.8 (Okamoto *et al.*, 1977). In the mitochondrial membrane F_o exposes one polypeptide at each side of the membrane (Ludwig *et al.*, 1980). In "inside out" ESMP F_o can directly sense, as in thylakoids, intravesicular pH changes. Acidification of this space can induce high H^+ conductivity by causing protonation of critical residue(s) of F_o.

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