Journal of Bioenergetics and Biomembranes, Vol. 16, No. 4, 1984

On the Mechanism of H^+ Translocation by Mitochondrial H^+-ATPase. Studies with Chemical Modifier of Tyrosine Residues

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Received September 22, 1983

Abstract

In this paper a detailed study of the effect of nitration of tyrosine residues by tetranitromethane on H^+ conduction and other reactions catalyzed by the H^+-ATPase complex in phosphorylating submitochondrial particles, uncoupled particles, and the purified complex is presented. Tetranitromethane treatment of submitochondrial particles results in marked inhibition of ATP hydrolysis, ATP–$^{33}P_i$ exchange, and proton conduction by the H^+-ATPase complex. These effects are caused by nitration of tyrosine residues of H^+-ATPase complex as shown by the appearance of the absorption peak at 360 nm (specific for nitrotyrosine formation) and inhibition of ATP hydrolysis and ATP–$^{33}P_i$ exchange in the complex purified from tetranitromethane-treated particles. H^+ conduction in phospholipid vesicles inlaid with F_0 is also inhibited by tetranitromethane treatment. These observations indicate that tyrosine residue(s) of F_0 are critically involved in energy-linked proton translocation in the ATP-ase complex.

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

Introduction

The mitochondrial H^+-ATPase complex is a membrane-associated enzyme which utilizes the electrochemical proton gradient, generated by the respiratory chain, to generate ATP and ADP and P_i (Mitchell, 1966, 1972). The complex can be resolved into two multipeptide moieties: the soluble catalytic sector F_1 and the membrane sector F_0 (Pedersen, 1975; Kagawa, 1978; Senior and Wise, 1983). It has been shown that F_0 functions as a transmembrane H^+ translocator in the native (see Papa and Guerrieri, 1981, for a review) and

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0145-479X/84/0800-0251$03.50/0

artificial phospholipid membranes (Okamoto *et al.*, 1977; Sigrist-Nelson and Azzi, 1980; Negrin *et al.*, 1980; Gasler *et al.*, 1980). In the mitochondrial H^+-ATPase there are additional polypeptide subunits: the oligomycin-sensitivity conferral protein (OSCP) which is apparently involved in the binding of F_1 to F_0, and an ATPase inhibitor protein (Senior and Wise, 1983).

Recently definite progress has been made in the elucidation of the protein structure of F_1 and F_0 (Senior and Wise, 1983). However, the mechanism of H^+ translocation through the enzyme is not yet known. As a contribution to understanding the molecular mechanism of H^+ translocation by H^+-ATPase, the characteristics of anaerobic relaxation of the electrochemical proton gradient $\Delta\mu H^+$ set up by respiration in submitochondrial particles, where F_1 is displaced or removed, have been studied (Pansini *et al.*, 1978, 1979; Guerrieri and Papa, 1981, 1982). In these particles the anaerobic relaxation of respiration-induced $\Delta\mu H^+$ takes place through the proton-conducting pathway in H^+-ATPase. Kinetic analysis of this process and studies with F_0 and F_1 inhibitors provided evidence that proton conduction in the H^+-ATPase is regulated by transmembrane $\Delta\mu H^+$ and by interactions between polar groups of F_1 and F_0 (Pansini *et al.*, 1978, 1979; Kopecky *et al.*, 1983).

Studies with amino acid modifiers indicated that arginine, tyrosine, and thiol groups of the membrane sector play a role in proton conduction by the H^+-ATPase of mitochondria (Guerrieri and Papa, 1981, 1982). In particular it has been shown that tyrosine nitration with tetranitromethane in sonic submitochondrial particles causes inhibition of H^+ translocation by the ATPase complex (Guerrieri and Papa, 1981).

In this paper a detailed study is presented of nitration of tyrosine residues in the H^+-ATPase complex and of its effect on H^+ translocation and other reactions catalyzed by the H^+-ATPase in phosphorylating submitochondrial particles, uncoupled particles, and purified ATPase complex.

Materials and Methods

Chemicals

Oligomycin and valinomycin were obtained from Sigma Co. (St. Louis, Missouri); phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, reduced β-nicotinamide adenine dinucleotide (NADH), adenosine-5-triphosphate, and catalase from Boehringer (Mannheim, FRG); and tetranitromethane from Aldrich Europe (Beerse, Belgium). All the other chemicals were of high-purity grade.

Preparation of Submitochondrial Particles

"Inside out" submitochondrial particles were obtained by exposure of beef heart mitochondria to ultrasonic energy in the presence of Mg-ATP (phosphorylating Mg-ATP particles, see Löw and Vallin, 1963) or of EDTA at pH 8.5 (uncoupled ESMP,[3] see Lee and Ernster, 1968). F_1-deficient particles (USMP) were prepared by urea treatment of ESMP as described by Horstmann and Racker (1970).

Isolation of the H^+-ATPase Complex

The H^+-ATPase, complex V, was prepared as described by Stiggall *et al.* (1978). F_0 was prepared as described by Galante *et al.* (1981).

Treatment of Submitochondrial Particles with Tetranitromethane

Tetranitromethane, diluted with ethanol 1:10 (v/v), was added, at the final concentrations given in the legend to the tables and 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 sedimented by centrifugation at 105,000 *g* for 10 min. The pellet was resuspended in 0.25 M sucrose and the particles, sedimented by centrifugation at 105,000 *g*, were collected in 0.25 M sucrose. The nitration of tyrosine residues in membrane proteins was verified by the appearance of the absorbance peak at 350–360 nm which is characteristics for nitrotyrosine (Guerrieri and Papa, 1981).

When desired, complex V was extracted from these particles and the presence of nitrotyrosine was verified by the appearance of the absorbance peak at 360 nm (Fig. 1).

Measurement of Proton Translocation

Submitochondrial particles, 3 mg of protein/ml, were incubated in a reaction mixture containing 0.25 M 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 was 1.5 ml at pH 7.5. Incubation was carried out in a glass vessel, under a constant stream of N_2, thermostated at 25°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

[3]Abbreviations: ESMP, EDTA submitochondrial particles; USMP, F_1-deprived sonic submitochondrial particles by urea treatment; DCCD, *N,N'*-dicyclohexylcarbodiimide.

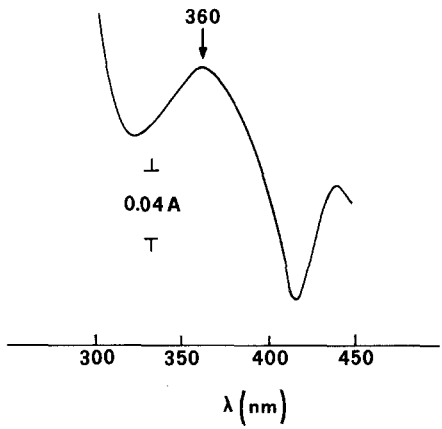

Fig. 1. Differential spectrum of complex V purified from tetranitromethane-treated Mg-ATP particles. For tetranitromethane treatment and purification procedure, see Materials and Methods. Complex V purified from tetranitromethane-treated submitochondrial particles (0.4 mg protein · ml^{-1}) was suspended in 1 ml of 0.1 M K-phosphate (pH 7.4). In the reference cuvette, complex V purified from untreated particles was present. The spectrum was recorded using a thermostated (25°C) spectrophotometer.

potentiometrically with a Beckman combination electrode (No. 39030, Beckman Instruments International, Geneva, Switzerland), connected to a Keithley Differential Electromer 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 anaerobic proton release from submitochondrial particles, the potentiometric traces were converted into proton equivalents by double titration with standard HCl and treated by a double exponential equation (Papa *et al.*, 1973).

Determination of ATPase Activity

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, 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 of lactate deydrogenase, 2 units of pyruvate kinase, and 20–30 μg protein of submitochondrial particles in a final volume of 1 ml.

Determination of ATP–$^{33}P_i$ Exchange

The incubation mixture contained 50 mM Tris/acetate (pH 7.5), 600 mM sucrose, 30 mM $MgSO_4$, 40 mM potassium phosphate (pH 7.4), and 6 mg/ml of bovine serum albumin. To 0.5 ml of this mixture, at 30°C, 10^6 cpm of $^{33}P_i$, water to bring the volume to 0.925 ml, and of 0.025 ml of a suspension of Mg-ATP particles (or complex V) containing 4 mg protein/ml in 0.05 M Tris/HCl, 0.66 M sucrose and 1 mM histidine (pH 8.0) and phospholipids (150 μg phosphorus/mg protein) were added. After 2 min preincubation the reaction was started by the addition of 0.05 ml of 0.24 M ATP, pH 7.0, and stopped after 5 min with 0.1 ml of 35% perchloric acid, followed by centrifugation in an Eppendorf centrifuge for 15 min at top speed. One-half milliliter of the deproteinized supernatant was used for analysis of $^{33}P_i$-labeled ATP as described by Stiggall *et al.* (1978). A separate 0.2-ml sample of the deproteinized reaction mixture was immediately immersed in liquid nitrogen for subsequent neutralization and determination of the amount of ATP remaining in the reaction mixture. For other details see Stiggall *et al.* (1978).

Preparation of F_0 Vesicles

For reconstitution experiments F_0 vesicles were prepared by the dialysis method as described in Okamoto *et al.* (1977). Proton conduction of the vesicles was measured potentiometrically and was induced by diffusion potential (positive inside) imposed by valinomycin-mediated potassium influx in the vesicles. For these measurements, vesicles corresponding to 0.15 mg of protein of F_0 were suspended in 1 ml of 0.15 M KCl, 20 mM $MgSO_4$, and 0.2 mM tricine, pH 8.0.

Results

Table I shows that tetranitromethane treatment of Mg-ATP particles results in inhibition of the ATPase activity and ATP–P_i exchange. The ATP–P_i exchange was more sensitive than the ATPase reaction; at 0.6 μmol of tetranitromethane per milligram of protein it was practically suppressed, while the ATPase activity was only 40% inhibited.

Figure 2 shows cycles of proton translocation induced by oxygen pulses of anaerobic ESMP supplemented with succinate as respiratory substrate. Tetranitromethane treatment resulted in a marked depression of the rate of anaerobic release of the protons taken up by ESMP during respiratory pulse, as indicated by the increase of the $t_{1/2}$ of the process from 1 to 1.9 sec. Addition of 0.4 μg/mg protein, oligomycin increased the $t_{1/2}$ of the process to 2.3 sec.

Table I. Effect of Tetranitromethane Treatment on ATPase and ATP–P_i Exchange Activities of Mg-ATP Sonic Submitochondrial Particles[a]

Additions	ATPase activity (nmol · min^{-1} · mg $protein^{-1}$)	Inhibition (%)	ATP–P_i exchange (nmol · min^{-1} · mg $protein^{-1}$)	Inhibition (%)
None	350		114	
Tetranitromethane (0.3 μmol · mg $protein^{-1}$	220	37	10	91
Tetranitromethane (0.6 μmol · mg $protein^{-1}$)	200	43	2	98

[a] For experimental conditions and tetranitromethane treatment, see Materials and Methods.

The inhibitory action of oligomycin was largely nonadditive with that exerted by tetranitromethane treatment.

As expected, the inhibition of proton backflow by oligomycin resulted in a significant enhancement of the extent of proton uptake at the aerobic steady state. The inhibitory effect of tetranitromethane treatment was, on the

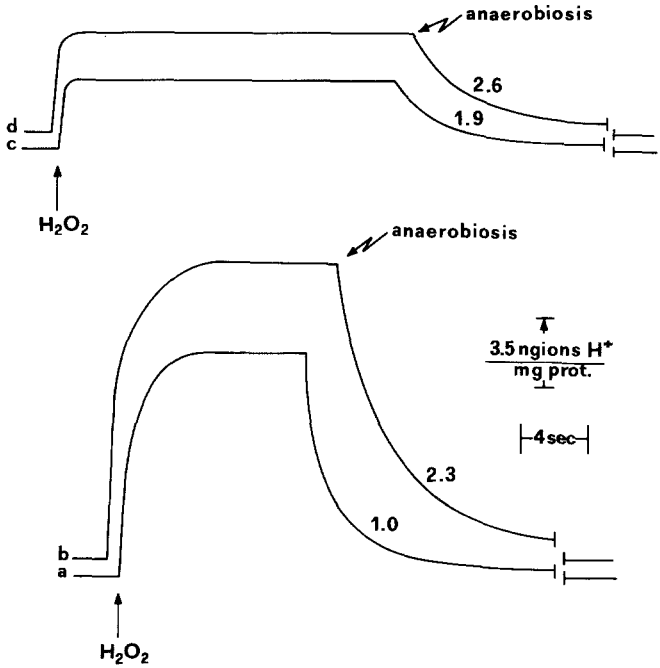

Fig. 2. Proton translocation induced by respiratory pulses in ESMP. For experimental procedure and tetranitromethane treatment, see Materials and Methods. (a) Control; (b) ESMP incubated in the reaction mixture for 10 min with oligomycin (0.4 μg · mg $protein^{-1}$); (c) ESMP treated with tetranitromethane (0.3 μmol · mg $protein^{-1}$); (d) as (c) + oligomycin (0.4 μg · mg $protein^{-1}$). The numbers reported on the pH traces refer to $t_{1/2}$ of proton release under anaerobiosis.

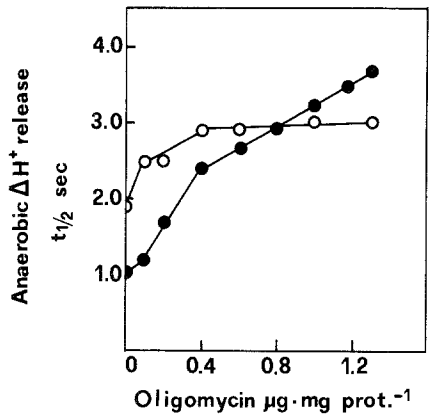

Fig. 3. Titration of the inhibition of oligomycin of anaerobic proton release from ESMP. For tetranitromethane treatment (0.3 μmol · mg protein^{-1}) and experimental procedure, see Materials and Methods. Additions: (●) none; (○) tetranitromethane (0.3 μmol · mg protein^{-1}).

contrary, accompanied by depression of the initial rate and of the extent of aerobic proton uptake by ESMP. This indicates that the treatment with the tyrosine modifier, besides inhibiting passive proton backflow, also caused depression of respiration-driven proton uptake in ESMP.

In Fig. 3 titration curves for the inhibitory effect of oligomycin on anaerobic H^+ relaxation from control ESMP particles and particles treated with tetranitromethane are shown.

It can be seen that the inhibitory efficacy of oligomycin was lowered by pretreatment of the particles with tetranitromethane. At the highest concentrations used, oligomycin caused a more profound inhibitory effect in the control than in the tetranitromethane-treated particles.

Similarly to that observed for proton conduction, treatment with tetranitromethane reduced also the inhibitory action of oligomycin on the ATPase activity of ESMP (Table II).

Treatment with tetranitromethane of particles deficient in F_1 (USMP) also resulted in inhibition of anaerobic relaxation of respiratory $\Delta\mu H^+$ (Guerrieri and Papa, 1981). The inhibitory action exerted by oligomycin on

Table II. Effect of Tetranitromethane Treatment of ESMP on the Oligomycin Inhibition of ATPase Activity[a]

Additions	Control	Inhibition (%)	+TNM (0.3 μmol · mg protein^{-1})	Inhibition (%)
None	350		220	
Oligomycin (0.5 μg · mg protein^{-1})	29	92	53	76
Oligomycin (1 μg · mg protein^{-1})	26	93	48	78

[a] For oligomycin-inhibited particles, incubation was carried out with the concentrations reported in the table for 5 min at 25°C The activity is expressed as nmol · min^{-1} · mg protein^{-1}.

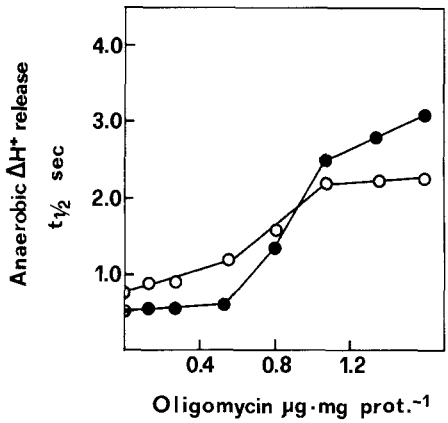

Fig. 4. Titration of the inhibition by oligomycin of anaerobic H^+ release from USMP. For experimental procedure and tetranitromethane treatment (0.3 μmol · mg protein^{-1}), see Materials and Methods. Symbols: (●) untreated USMP; (○) tetranitromethane-treated USMP.

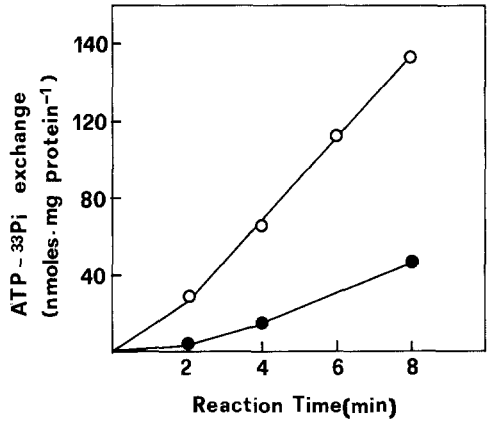

Fig. 5. Effect of tetranitromethane treatment on ATP–$^{33}P_i$ exchange activity of complex V. For experimental procedure and isolation of complex V from tetranitromethane-treated particles, see Materials and Methods. Symbols: (○) complex V isolated from Mg-ATP particles; (●) complex V isolated from tetranitromethane-treated Mg-ATP particles (0.3 μmol · mg protein^{-1}).

anaerobic H^+ release gives a sigmoidal titration curve. Also in this case tetranitromethane reduced the inhibitory activity of oligomycin (Fig. 4).

Figure 5 shows the time course of ATP–$^{33}P_i$ exchange in complex V prepared by control and tetranitromethane-treated Mg-ATP particles. It can be seen that in the latter preparation the ATP–P_i exchange activity was severely inhibited (Fig. 5). Also the ATPase activity was markedly inhibited in complex V from tetranitromethane-treated particles as compared to the control complex (Fig. 6). As in the case of submitochondrial particles (see Table I) the ATPase activity was, however, less sensitive to tetranitromethane treatment than ATP–P_i exchange. Furthermore, it can be noted that the

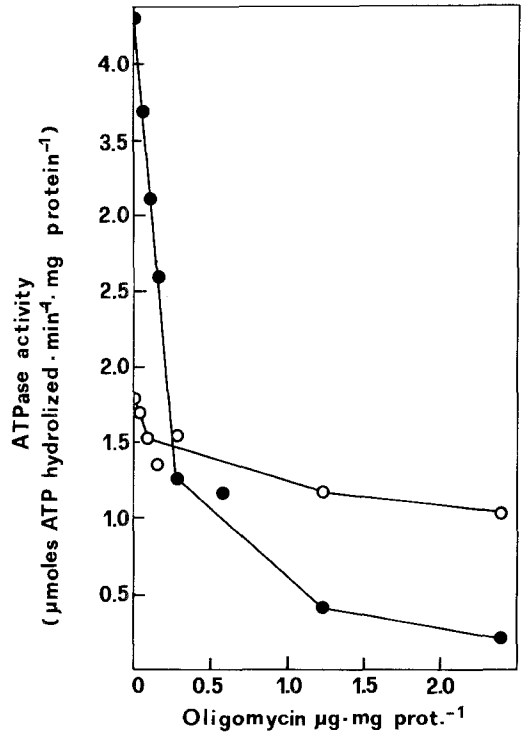

Fig. 6. Effect of oligomycin on ATPase activity of purified complex V from Mg-ATP particles. For purification of complex V and tetranitromethane treatment, see Materials and Methods. For measurement of ATPase activity, complex V (50 $\mu g \cdot ml^{-1}$) was incubated as described by Stiggall *et al.* (1978). Symbols: (●) complex V isolated from Mg-ATP particles; (○) complex V isolated from particles treated with 0.3 μmol of tetranitromethane per milligram protein.

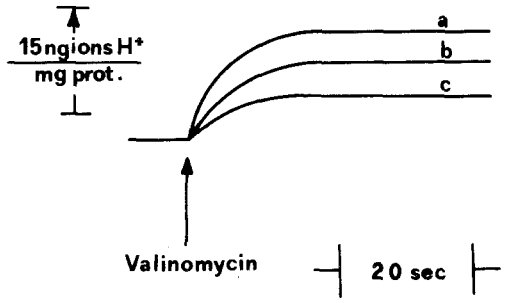

Fig. 7. Inhibition by tetranitromethane of proton conduction of F_0 inlaid in liposomes. For preparation of F_0, see Galante *et al.* (1981). The reconstitution was carried out as described by Okamoto *Et al.* (1977). For amino acid residues modification, F_0 vesicles were treated with modifiers at room temperature for 30 min. After this interval the vesicles were spooned down by centrifugation at 105,000 *g* for 20 min. Then the pellet was suspended in a solution containing 0.15 M KCl, 20 mM sucrose, 5 mM $MgSO_4$, and 0.2 mM tricine, pH 8.0; the final concentrations of F_0 was 0.15 mg protein/per ml. A sample of 2 ml was taken for the determination of H^+ translocation. Additions: trace a, none; trace b, tetranitromethane 0.3 μmol · mg protein^{-1}; trace c, *N*,*N*′-dicyclohexylcarbodiimide 80 nmol · mg protein^{-1}.

ATPase activity of complex V from tetranitromethane-treated particles exhibited a decreased sensitivity to oligomycin (Fig. 6).

Figure 7 illustrates the proton release from liposomes inlaid with purified F_0, induced by valinomycin-mediated K^+ influx. Proton release in the reconstituted system was inhibited by DCCD and tetranitromethane treatment similarly to that observed in submitochondrial particles.

SDS/PAGE patterns (Weber and Osborn, 1976) of tetranitromethane-treated particles (or complex V) were similar to those of untreated particles (or complex V) (not shown), indicating that modification by tetranitromethane treatment did not cause any gross modification of the subunit pattern of the complex.

Discussion

Tetranitromethane treatment of submitochondrial particles causes inhibition of ATP hydrolysis, ATP–P_i exchange, and proton conductivity of the H^+-ATPase complex.

The H^+-ATPase complex isolated from tetranitromethane-treated par-

ticles shows the absorbance peak at 360 nm characteristic of nitrotyrosine formation (Fig. 1), as well as inhibition of ATP hydrolysis and ATP–P_i exchange (Figs. 6 and 7). These results indicate that, under the conditions used, tetranitromethane treatment of sonic submitochondrial particles results in nitration of tyrosine residue(s) in the H^+-ATPase complex.

It has been reported that tetranitromethane modifies tyrosine residue(s) in F_1 (Senior, 1973). The present study shows that tetranitromethane modifies also critical tyrosine residues in F_0. In fact, treatment with this reagent of F_1-depleted submitochondrial particles (USMP) or of F_0 reconstituted into phospholipid vesicles causes an inhibition of proton conductivity as effective as that observed after treatment of F_1–F_0 particles (ESMP).

Tetranitromethane treatment of ESMP or USMP results in a lower sensitivity of proton conduction to inhibition by oligomycin.

Evidence has been obtained showing that oligomycin interacts with hydrophobic residues in the DCCD-binding 8-kDa polypeptide of F_0 (Hoppe and Sebald, 1981) close to a glutamic residue critical for proton conduction. It is conceivable that tetranitromethane modifies tyrosine residues in this polypeptide in a segment close to that effected by oligomycin and DCCD.

The present observations would therefore support the possibility that tyrosine and other hydroxyl residues in the 8-kDa polypeptide participate in proton conduction possibly by constituting a proton-conducting pathway in F_0 of the type proposed by Nagle and Morowitz (1978). The inhibition of the ATP–$^{33}P_i$ exchange caused by tetranitromethane treatment of submitochondrial particles or isolated ATPase complex is much more marked than the inhibition of the hydrolysis of ATP and is as extensive as the inhibition of proton conductivity. This indicates the close functional association of proton translocation with the ATP–P_i exchange, which is, in fact, considered as an index of the coupling capacity of the ATPase complex (Stiggall *et al.*, 1978; Kagawa and Racker, 1971).

Acknowledgments

The authors are indebted to Dr. Y. Hatefi for discussion and suggestions.

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