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On the Relationship between Mitochondrial ATPase and the Inner Membrane

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Abstract

Digestion of the submitochondrial particle (ETP_H) with a proteolytic enzyme, Nagarse, selectively and completely removed the headpieces from the membrane without damaging the electron transfer chain. By determining the amount of protein released by the Nagarse treatment, it was calculated that the headpieces represent $16 \pm 0.5\%$ of the total protein of the submitochondrial particles.

In respiring ETP_H, membrane-bound AMP was found to be an acceptor of inorganic phosphate, and this esterification led to the formation of membrane-bound ADP. About 70% of the membrane-bound adenine nucleotides were found to be tightly bound to the intrinsic proteins of the membrane. A transphosphorylation reaction was observed between external and membrane-bound ADP.

Introduction

The structural and functional organization of the mitochondrial ATP synthesizing system and the electron transfer system is an important contemporary problem. There is general agreement, based on reports from many laboratories (notably those led by Green and Racker) that the electron-transfer complexes and the ATPase enzyme (Racker's F_1*) represent respectively the sites of oxido-reduction and ATP synthesis. The ATPase is the headpiece of the TRU discovered by Fernández-Morán *et al.* [1] and the electron-transfer complexes are located in the basepieces. The cylindrical stalk of the TRU which has been identified as

* Abbreviations: F_1, coupling factor one; OSCP, oligomycin-sensitivity conferring protein; TRU, tripartite repeating unit; ETP_H, phosphorylating electron transfer particle.

the OSCP [2, 3] is the structural connection between the headpiece and the basepiece.

A knowledge of the mass contributed by the headpiece and the stalk to the total protein of the inner membrane can serve as a key for elucidating the fundamental structure of the electron transfer and ATP synthesizing system as well as for defining the interaction between these two systems. In the earlier literature [4], it was suggested that there is 1 : 1 molar relationship between the headpiece-stalk and the electron-transfer complex. This implies that the headpiece represents over 30% of the protein mass of the total inner membrane. On the other hand, Kagawa and Racker [5] estimated that F_1 represents 5 to 10% of the total mitochondrial protein. This estimate, which was based on the examination of electron micrographs of submitochondrial particles, can hardly be considered very reliable because of the uneven distribution of the headpiece-stalk projections on the surface of the inner membrane.

We have found that Nagarse, a bacterial proteinase, can selectively and completely digest the headpieces of ETP_H with concomitant complete loss of ATPase activity. This treatment does not damage the electron-transfer chain. We have obtained an accurate estimate of the contribution made by the headpiece to the total protein of the inner membrane by utilizing a selective digestion technique. This paper also deals with the phosphorylation of the adenine nucleotides bound in the inner membrane.

Materials and Methods

Adenine Nucleotide. ADP was supplied as its sodium salt by Sigma Chemical Company. Crystalline AMP was supplied by Pabst Laboratory. The concentration of nucleotide was determined by measurements of absorbancy at 260 mμ using the millimolar extinction coefficients of 15.0 x cm^{-1} at pH 7.0 [6] and 13.5 x cm^{-1} in formic acid. [7]

Other Chemicals. Radioactive phosphoiric acid ($^{32}P_i$) was supplied by International Chemical and Nuclear Corporation, and was used after hydrolysis in HCl. The ion-exchange resin, Dowex-1 x-8, 200 ~ 400 mesh, was supplied by Calbiochem. in its formate form.

Enzymes. Nagarse, a crystalline bacterial proteinase, was supplied by Teikoku Chemical Industry Company (1500 P.U.N./mg protein). The source of phospholipase A was an extract of the boiled venom of *Crotalus atrox* from the Ross Allen Reptile Institute. The venom suspension was heated for 5 min in a boiling water bath. The boiled extract, pH 5.0, was centrifuged for 10 min at 105,000 x g. The clear supernatant fluid was collected and adjusted to pH 7.0 with 1 M Tris-chloride. The final protein concentration was about 1 mg/ml. 5′-Adenylic acid deaminase (1 μmolar unit/0.57 ml) and hexokinase

(270 units/mg protein) were supplied by Sigma Chemical Company. Phosphoglycerate kinase, glyceroaldehyde dehydrogenase, pyruvate kinase and adenylate kinase were supplied by Boehringer Mannheim.

Submitochondrial Particles. ETP_H was prepared from beef heart mitochondria by the method of Hansen and Smith [8] The particles were washed by centrifugation with a solution which was 0.25 M in sucrose and 10 mM in Tris-acetate, pH 7.5, and were suspended in the same medium.

Electron Microscopy. The specimens were prepared for electron microscopy by negative staining with phosphotungstic acid [9]. A drop of the particle suspension, usually at a protein concentration of 0.5 mg/ml, was placed on a carbon-coated copper grid. Excess solution was removed with filter paper and the samples were allowed to dry. All specimens were examined in a Hitachi type HU 11-B electron microscope operated at 75 kV.

Assay for the Enzymic Activity. ATPase activity was measured by estimating P_i released from ATP after separation of P_i from the organic phosphate by the method of Lindberg and Ernster [10]. Alternatively, it was measured by the H^+ change accompanying hydrolysis of ATP. The measurements were performed by tracing the pH change accompanying ATP hydrolysis with a Beckman's expanded scale pH meter. The amount of H^+ released was calibrated by adding a standard HCl solution to the reaction mixture. The rates of oxidation of NADH and succinate in the particles were measured at 25°C under the conditions described by Blair *et al.* [11].

Separation and Analysis of Membrane-bound Adenine Nucleotide. Bound adenine nucleotide was extracted from the particles by perchloric acid. The solution of nucleotides was mixed with carrier nucleotide and the mixture was absorbed onto charcoal. Inorganic phosphate was washed out with 1% perchloric acid. The nucleotides were eluted from charcoal with a solution which was 50% in ethanol and 2% in ammonia: the eluate was evaporated to dryness. The nucleotides were separated from one another on a Dowex-1 formate column [12]. ATP in the sample was also determined enzymatically with the combination either of hexokinase and glucose-6-phosphate dehydrogenase or of phosphoglycerate kinase and glyceroaldehyde dehydrogenase (Boehringer's ATP test kit). ADP and/or AMP were measured with the combination of pyruvate kinase and lactate dehydrogenase with or without adenylate kinase. AMP was also measured with 5′-adenylate deaminase [13].

Results

Removal of the Headpiece from the Membrane by Enzymic Treatment. We attempted to split the headpieces off the membranes by treating

ETP_H with Nagarse or phospholipase or with a combination of the two. ETP_H was suspended in a medium 0.25 M in sucrose and 10 mM in Tris-acetate, pH 7.5, (sucrose-Tris) at a concentration of 20 mg protein/ml. To 3 ml of the ETP_H suspension were added 50 μmoles of K_2HPO_4, 10 μmoles of $CaCl_2$, 1 μmole of potassium EDTA, and 1.7 ml of the sucrose-Tris medium to a total volume of 5 ml. The control (S_2) contained no enzyme. Crystalline Nagarse (60 mg protein) was added to one of the experimental mixtures (S_3). To another experimental mixture (S_4), phospholipase A (1 mg protein) was added. The same amounts of both Nagarse and phospholipase A were added to a fourth experimental mixture (S_4). The suspensions of both S_1 and S_2 were incubated at 22°C for 5 min, whereas those of both S_3 and S_4 were incubated for 30 min. After the incubation, the particles were washed twice by centrifuging through a medium 0.25 M in sucrose and 10 mM in Tris-acetate, pH 7.5, at 150,000 x g for 30 min. The washed particles in each run were suspended in 3 ml of the sucrose-Tris medium and the suspensions were homogenized by brief sonication.

ATPase activity in the treated particles was measured either by determination of P_i or by tracing the acid formation accompanying the hydrolysis of ATP in a reaction mixture which was 0.25 M in sucrose, 10 mM in Tris-acetate, pH 7.5, 5 mM in $MgCl_2$, 0.5 mM in EDTA and 10 mM in ATP. A typical trace of the proton release due to ATPase activity is shown in Fig. 1. The Nagarse treated sample (S_2) showed no activity, whereas the phospholipase treated sample (S_3) showed activity comparable to that of the control (S_1). The sample containing both Nagarse and phospholipase (S_4) showed no ATPase activity.

Electron micrographs of these particles are shown in Fig. 2. The headpiece and stalk are clearly observable in the control and phospholipase-treated particles (A and C in Fig. 2). The headpieces are absent, but the stalks are visible on the surface of the particles treated with Nagarse (B in Fig. 2). The incubation of the particles with Nagarse and phospholipase resulted in the complete removal of both the headpiece and the stalk from the membrane (D in Fig. 2). Phospholipase-treated particles show clearer visualization of the detachable sector (headpiece and stalk) than that of the control particles. From the photographs, we deduce that phospholipase treatment leads to the flattening of the vesicles. Our interpretation of the electron microscopic results is represented in Fig. 3. The observations strongly suggest that Nagarse attacks the junction between the headpiece and the stalk and this results in the complete removal of ATPase activity from the particles.

Weight Contribution of the Headpiece. To estimate the exact contribution made by the headpiece to the protein mass of the inner membrane, ETP_H was treated with varying amounts of Nagarse for 5 min at 22°C. The treated particles were washed twice with the sucrose-Tris

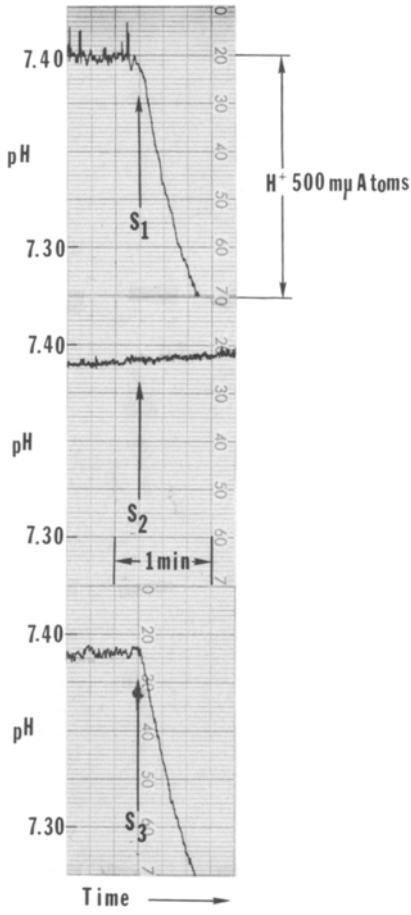
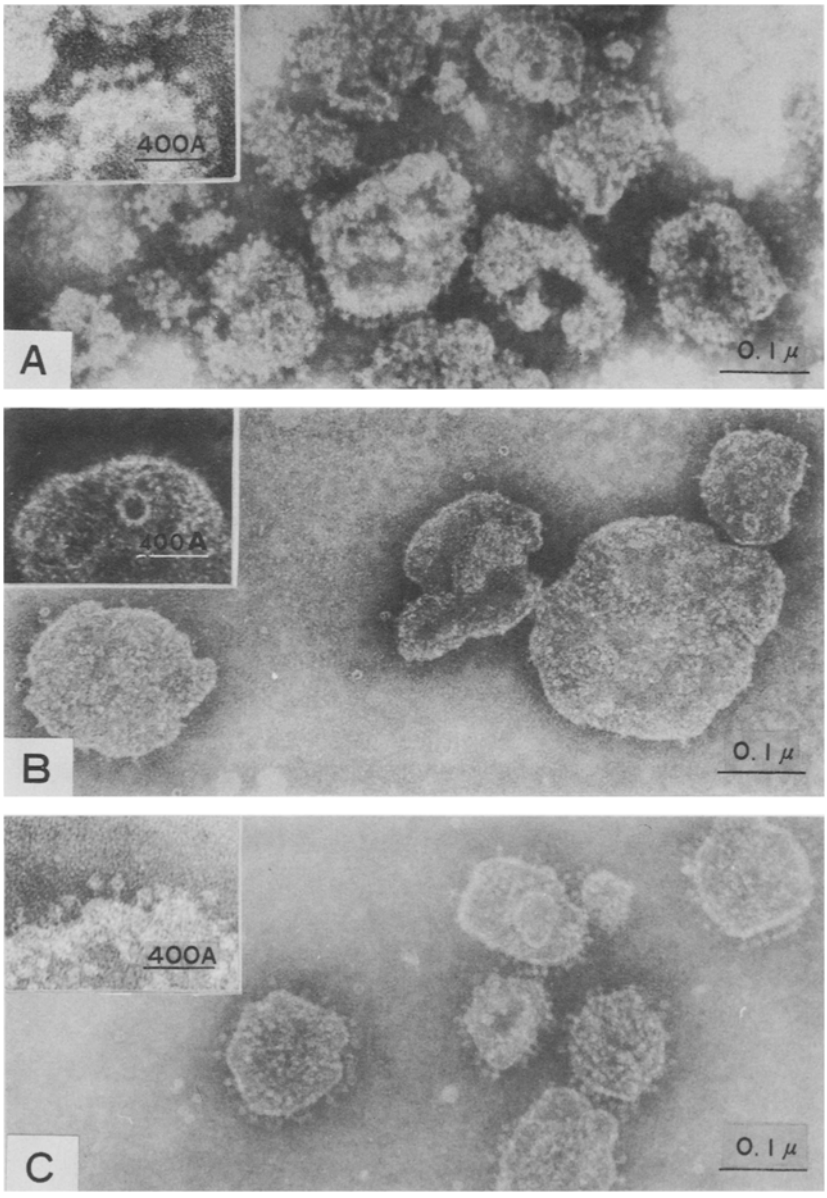


Figure 1. Removal of ATPase activity in Nagarse-treated submitochondrial particles. ETP_H were treated with Nagarse or with phospholipase, and were washed twice as described in the text. To the reaction mixture, which was 0.25 M in sucrose, 10 mM in Tris-acetate, pH 7.5, 5 mM in $MgCl_2$ and 0.5 mM in EDTA, the particles were added where indicated by the arrows at a concentration of 1 mg protein/ml, at 22°C. The proton release due to ATP hydrolysis was recorded. S_3, control not exposed to enzymic treatment. S_2, experimental exposed to Nagarse. S_3, experimental exposed to phospholipase.

medium, suspended in the same medium and the suspension was homogenized by brief sonication. The protein concentration of the treated particles was determined by the biuret method [14] standardized with crystalline bovine serum albumin. This method was tested and shown to be in good agreement with the dry weight method introduced by Slater [15].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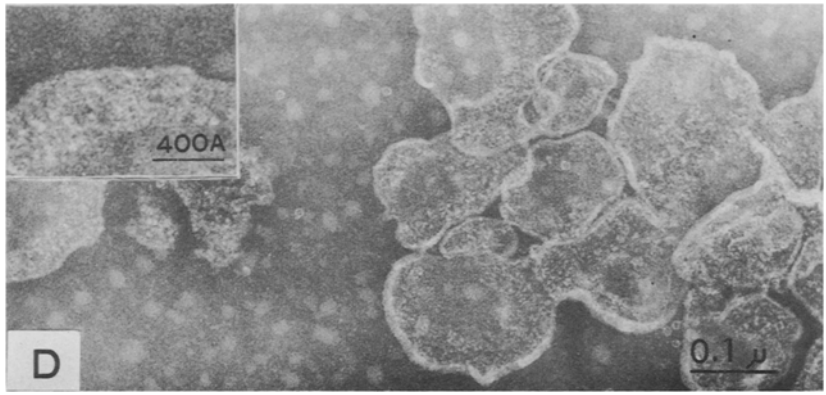

Figure 2. Electron micrographs of negatively stained ETP_H. A: The control suspension which was incubated without enzymic treatment. B: The experimental treated with Nagarse. C: The experimental treated with phospholipase. D: The experimental treated with both Nagarse and phospholipase. Magnification: x15,000. Inserts: x230,000. Ferritin was added to the preparation as an internal marker of size.

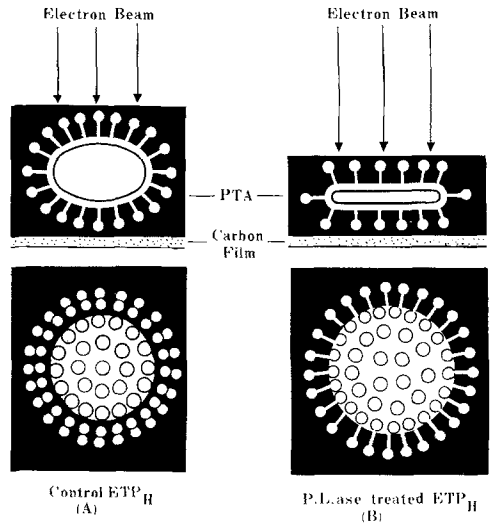

Figure 3. A diagrammatic interpretation of the state of the detachable sectors in particles treated in the various fashions described in the legend to Fig. 2. Submitochondrial particles treated with phospholipase show clearer visualization of the "stalk" than the control particles because of the flattening of the vesicles induced by phospholipase treatment. Top: The relation of the particles stained with phosphotungstate on the grids to the electron beam (side view). Bottom: Projections of the particles on the screen of the electron microscope (top view).

The results of the enzymatic analyses of the Nagarse treated particles are summarized in Table I. In good agreement with the decrease in ATPase activity, the particles lose up to 16% of the original to tal protein. On the other hand, the original activity of both NADH- and succinate oxidase was fully retained in the treated particles, and the respective specific activities were increased to a degree compatible with the amount of inert protein released. The results clearly show that Nagarse preferentially attacks the ATPase (headpiece) but not the electron transfer chain which is located in the basepieces. The headpiece thus represents 16% of the protein content of the original particle.

TABLE I. ETP_H after exposure to Nagarse

Nagarse added mg/mg ETP_H of protein	Total membranous protein mg	Oxygen consumed NADH Succinate μatoms/min/mg of protein		ATP hydrolyzed μmoles/min/mg of protein
0	60.0 ± 0.8	1.42	0.10	0.92
0.10	56.6 ± 0.8	1.52	0.10	0.42
0.25	52.8 ± 0.6	1.60	0.11	0.27
0.50	51.0 ± 0.6	1.65	0.12	0.08
1.00	50.4 ± 0.6	1.70	0.12	<0.00

ETP_H (5 ml; 60 mg) suspended in a medium 0.25 M in sucrose and 10 mM in Tris-acetate of pH 7.5, was incubated with each of the indicated amounts of Nagarse for 5 min at 22°C. The particles were washed twice by centrifuging through the sucrose-Tris, suspended into 3 ml of the sucrose-Tris and homogenized. Protein concentration, NADH oxidase, succinate oxidase and ATP hydrolyzing activities were measured (see text). The values in the table represent the mean of six measurements with the corresponding standard error.

Location of Membrane-bound Nucleotides. It has been reported that electron transfer is coupled to the esterification of the bound adenine nucleotides of the inner membrane [16, 17]. To evaluate the role of the membrane-bound adenine nucleotides, the following experiments were carried out. ETP_H was preincubated for 2 min at 30°C in a medium which was 0.25 M in sucrose, 10 mM in Tris-acetate, pH 7.5, and 4 mM in $MgCl_2$. The reaction was initiated by addition of succinate and $^{32}P_i$ (specific activity of 30,000 cpm/nmole) to a final concentration of 10 mM. After an incubation period of 15 sec at 22°C when the phosphorylation of the bound nucleotides reaches a plateau, the respiration of the particle was stopped by addition of antimycin (10 μg/mg protein) and of Na_2S (0.3 mM). Then the particles were washed by centrifugation through a medium 0.85 M in sucrose, 10 mM in Tris-acetate, pH 7.5, and 0.3 mM in Na_2S. The washed particles were

suspended in a medium 0.25 M in sucrose and 10 mM in Tris-acetate, pH 7.5, at a concentration of 20 mg protein 1 ml. A part of the particles thus prepared was treated with perchloric acid to extract membrane-bound nucleotides and esterified ^{32}P. The results showed that in a respiring particle, $^{32}P_i$ was esterified and incorporated into membrane-bound nucleotides in an amount of 1.02 nmoles per mg protein under the conditions described above. Thus, about 50% of the membrane-bound nucleotides in the particles were labelled with ^{32}P.

The ^{32}P-labelled particles were then treated either with Nagarse or phospholipase or with both for 30 min at 22°C under the conditions described in the preceding section (see Table II). The major part of the esterified ^{32}P, about 70% of the total, was tightly bound to the particles.

TABLE II. Retention of bound nucleotides in ETP_H after exposure to proteolytic or phospholipalytic action

Additions	Esterfield ^{32}P bound in ETP_H and in treated derivative particles after washing nmoles/mg protein
None	0.70
AMP	< 0.70
ADP	< 0.00
ATP	0.20
Nagarse	0.65
Phospholipase	0.66
Nagarse + Phospholipase	0.64

ETP_H were incubated with $^{32}P_i$ and succinate for 15 sec at 30°C. The membrane-bound nucleotides were labelled with esterified ^{32}P, 1.02 nmoles/mg protein. ^{32}P-labelled particles were incubated for 30 min at 22°C with the additions indicated, then were spun down by centrifuging for 20 min at 150,000 x g. The nucleotides were extracted from the particles with perchloric acid, and esterified ^{32}P in the nucleotide fractions was counted.

Neither Nagarse, phospholipase nor the combination of the two could release the tightly bound esterified ^{32}P to any significant degree. Since the treatment of the particles with Nagarse and phospholipase completely removed the headpiece and the stalk from the membrane (Fig. 2), it is evident that the tightly bound esterified ^{32}P is located in the basepieces of the tripartite repeating unit, probably in a hydrophobic domain which is resistant to the action of the proteolytic enzyme. A part of esterified ^{32}P, about 30% of the total, was extracted after incubation of the particles for 30 min in a buffer with no addition of either Nagarse or phospholipase indicating its loose association with the particles.

Phosphoryl Transfer Between Membrane-bound and External Adenine Nucleotides. The ^{32}P labelled particles were incubated for

30 min at 22° C with externally added adenine nucleotides in place of the proteolytic enzymes. It was found that in the presence of externally added ADP or ATP, but not AMP (each 10 mM), the tightly bound esterified ^{32}P is almost completely removed from the particles as shown in Table II. Under these conditions, neither externally added ^{14}C-ADP nor ^{14}C-ATP can exchange with the membrane-bound adenine nucleotides. Esterified ^{32}P thus obtained from the ^{32}P-labelled particles was recovered and analyzed by Dowex-1 column chromatography. Enzymic determination of the adenine nucleotides was also carried out as described in Methods. The results in Table III show that ^{32}P is associated with ATP. Thus, the tightly bound ^{32}P in the basepieces transphosphorylates with external ADP to form external ATP.

TABLE III. Distribution of esterified ^{32}P among extraparticulate adenine nucleotides

Fraction of adenine nucleotides	% ^{32}P Label in the fraction	
	Exp. 1	Exp. 2
ADP	12	15
ATP	88	85

In Exp. 1, ^{32}P-labelled particles which were treated with Nagarse for 5 min as described in Table II, were suspended in a medium 0.25 M in sucrose and 10 mM in Tris-acetate, pH 7.5, at a concentration of 20 mg/ml, and were incubated with 10 mM ADP. After incubation for 5 min at 22° C, the particles were spun down. The nucleotides in the supernatant fluid were separated on a Dowex-1 column and the esterified ^{32}P in each fraction were counted.

In Exp. 2, the conditions were the same as in Exp. 1, except that ^{32}P-labelled particles were not treated with Nagarse, and were incubated with 16 mM fluoride as well as with ADP.

The membrane-bound nucleotides in ^{32}P labelled particles were extracted with perchloric acid and fractionated on Dowex-1 column as described in Methods. The distribution of ^{32}P in the extracted nucleotides is shown in Fig. 4. The esterified ^{32}P in the ADP fraction represented 90% of the total whereas the ATP fraction accounted for the remainder of the label. The data suggest that membrane-bound AMP is the precursor of bound AD^{32} P. The kinetic data which supports this conclusion have been published [17].

Discussion

Structural Organization of the Mitochondrial Inner Membrane. Electron microscopic observation as well as chemical evidence clearly demonstrate that Nagarse selectively detaches the headpiece (F_1) from the inner membrane (Figs. 1-3). A combination of Nagarse and phos-

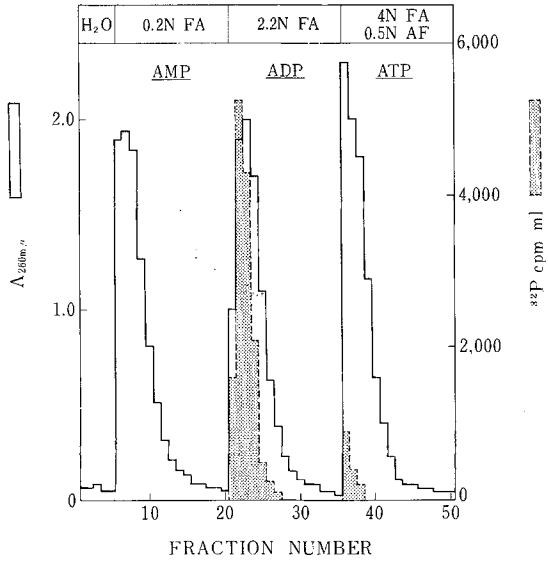

Figure 4. Distribution of esterified ^{32}P among the membrane-bound nucleotides in ETP_H. The membrane-bound nucleotides in ETP_H, which were labelled with ^{32}P during succinate-driven phosphorylation, as described in the text, were extracted with perchloric acid. Carrier nucleotides, AMP, ADP and ATP were added to the perchloric acid extract. They were separated from each other on Dowex-1 column as described in the text. Absorbancy at 260 mμ (A_{260} mμ) and ^{32}P radioactivity (cpm : ml^{-1}) of each fraction were measured. FA and AF in the figure stands for formic acid and ammonium formate, respectively.

pholipase detaches the headpiece-stalk sector. The amount of protein released from ETP_H after incubation with varying amounts of Nagarse reaches a maximum of 16% of the total inner membrane protein (see Table I).

Since the limited digestion of submitochondrial particles with Nagarse reported in this paper results in the complete removal of the headpiece and of ATPase activity whereas the total electron transfer activity is retained in the particles (Table I), we may conclude that the protein detached from the membrane representing 16 ± 0.5% of the protein of the inner membrane can be identified as the headpiece. The most recent evidence indicates that F_1 has a molecular weight of 347,000 [18]. On the basis of this molecular weight, one unit of the inner membrane which includes one headpiece, stalk and the corresponding membrane sector should have a "molecular" weight of $2.17 \pm 0.05 \times 10^6$. This value implies that the unit of inner membrane which combines with one headpiece consists of 6-8 complexes of average molecular weight 200,000 ~ 300,000.

Kagawa and Racker [5] calculated that F_1 represents between 5 and 10% of the total mitochondrial weight by inspection of electron micrographs of submitochondrial particles and estimation of the probable distribution of inner mitochondrial spheres. They concluded that F_1 represents about 7% of the protein of submitochondrial particles, based on a comparison of the ATPase activity in trypsin-treated particles and the specific activity of purified ATPase. However, the value, 7%, is a minimal one, since as they indicated, trypsin treatment may result either in incomplete activation or in partial inactivation of ATPase activity, or it may lead to both results.

Phosphorylation of Membrane-bound Nucleotides. It must be emphasized that there is a low but definite amount of phosphorylation of membrane-bound adenine nucleotides occurring, that is, associated with respiration [16, 17]. Although the role of membrane-bound adenine nucleotides has not been thoroughly clarified with respect to oxidative phosphorylation, these nucleotides might have a close relationship with the total phosphorylation process in the mitochondrion or in submitochondrial particle. The phosphorylation of the membrane-bound adenine nucleotides takes place in the membrane phase and the amounts involved are low (ca 1 nmole/mg protein). This reaction of bound nucleotide has to be distinguished from the phosphorylation of free adenine nucleotides both in the matrix and in the extra-mitochondrial space.

The evidence presented here as well as previously reported evidence [16, 17] indicate that (a) during respiration, membrane-bound AMP is an early acceptor of incoming phosphate and forms membrane-bound ADP; (b) about 70% of the membrane-bound adenine nucleotides seems to be tightly bound in the membrane phase and (c) a transphorylation reaction takes place between ADP in the membrane phase and external ADP.

There are two possibilities to be considered for the overall oxidative phosphorylation process: (a) if the phosphorylation of the membrane-bound adenine nucleotides is the primary event, then the phosphorylation of nucleotides in the matrix space is a secondary process or (b) if the phosphorylation of matrix nucleotides is the primary event, then the phosphorylation of the membrane-bound nucleotides is secondary. The former possibility is supported by the most recent evidence of Roy and Moudrianakis [19] and of Forti *et al.* [20] that the coupling factor from chloroplasts (CF_1) synthesizes enzyme-bound ADP from bound AMP in a light-driven reaction.

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