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STUDIES ON THE STABILIZATION OF AN OXIDATIVE PHOSPHORYLATION SYSTEM

I. RESISTANCE OF A PHOSPHORYLATING SYSTEM OF SUBMITOCHONDRIAL PARTICLES TO TRYPSIN, DUE TO PHOSPHORYLATION OF ADP

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SUMMARY

The action of trypsin on the phosphorylating system of submitochondrial particles was studied under various conditions.

The rate of proteolysis of the phosphorylating system did not decrease when the particles, acted upon by trypsin, simultaneously oxidized some substrate (NADH or succinate). The phosphate acceptor (ADP) itself did not stabilize the phosphorylating system either. The latter became definitely resistant to trypsin only under conditions favouring oxidative phosphorylation (*i.e.* in the presence of a substrate, oxygen, ADP and phosphate), or after treatment by ATP. The protective effect was manifested when measuring the P/O ratio, the rate of the ATP–$^{32}P_i$ exchange reaction and the rate of the ATP-dependent NAD^+ reduction by succinate. This effect depended on the rate and efficiency of the functioning of the oxidative phosphorylation system. Stabilization was maximal when the rate of NADH oxidation was 30–50 nmoles/min per mg of protein, and the P/O ratio was not lower than 1.9–2.0. The stabilizing action of succinate and ADP vanishes when oleate is added in the concentration necessary for complete uncoupling of respiration and phosphorylation. These facts indicate that under definite conditions stabilization of a phosphorylating system is a result of its functioning.

An assumption is advanced according to which the composition and structural organization of mitochondria are such as to ensure maximum resistance of the phosphorylating system to proteolysis.

INTRODUCTION

Mitochondria capable of existing for a long time in the active form *in vivo* are known to be very unstable *in vitro*. There are grounds for thinking that one of the main reasons for the degradation of mitochondria is the action of mitochondrial

phospholipase A[1–4] and endogenous proteinases[5]. It is also necessary to take into account the effects of lytic enzymes of lysosomes[6,7], usually contained in a certain amount in the mitochondrial fraction. If this is so, then to prevent the aging of mitochondria methods of increasing their resistance to proteolysis and the action of phospholipases have to be found. This refers primarily to the oxidative phosphorylation system, which is the main structural and functional unit of crystal membranes.

Several years ago we showed that under conditions where NADH or succinate are being oxidized by oxygen, the respiratory chain acquires a certain thermal stability and resistance to the action of trypsin (or chymotrypsin) and cobra venom phospholipase[8–12]. In this work the effect of trypsin on various functions of a phosphorylating system of submitochondrial particles is studied. The data obtained are evidence of the fact that ADP phosphorylation is accompanied by specific conformational changes in the phosphorylating system, which manifest themselves as a decrease in the rate of its proteolysis. Evidently, only the oxidative phosphorylation system of intact mitochondria, whose enzymatic composition and structural organization ensure continuous electron transfer in the respiratory chain and the ATP synthesis coupled to it, possesses high resistance to proteolysis.

MATERIALS AND METHODS

Preparation of submitochondrial particles

The submitochondrial particles, electron transport particles (ETP_H) (Mg^{2+}, Mn^{2+}) were isolated from heavy beef heart mitochondria[13]. Freezing and thawing of the mitochondria were carried out according to VALLIN[14]. The mitochondria were desintegrated according to BEYER[13]. For ultrasonic particles the P/O ratio was 2.0–2.6 when NADH was used as the substrate, or 1.1–1.7 when the substrate was succinate.

Assays

(1) *P/O ratios* were assayed according to SCHATZ AND RACKER[15], using radioactive orthophoshpate. The latter was first purified as described in ref. 16. The only deviation from SCHATZ AND RACKER[15] was that the orthophosphate was removed after deproteinization by double extraction with an isobutanol–benzene mixture (1:1, by vol.), and then with water-saturated isobutanol. This treatment resulted in complete removal of residual orthophosphate.

The assay medium contained 2 mM $MgCl_2$, 10 mM potassium phosphate (pH 7.4; $^{32}PO_4^{3-}$, $0.5 \cdot 10^5$–$1.5 \cdot 10^5$ counts/min per μmole of PO_4^{3-}), 33 mM glucose, 0.5 mg bovine serum albumin per ml, 0.5 mM EDTA, 2.0 mM ATP (pH 7.4), 0.25 mg yeast hexokinase per ml, 5 mM Tris–HCl (pH 7.4), 120 mM sucrose, and 16 μg soya trypsin inhibitor per ml. The succinate concentration was 7 mM. NADH was generated by a system consisting of 30 mM ethanol, alcohol dehydrogenase (100–200 μg/ml), 0.2 mM NAD^+, and 6 mM semicarbazide. In this case the reaction was started by introducing ethanol. The particle concentration was 0.6 mg protein per ml (final volume 1.4 ml).

Assays were carried out at 35°. Special experiments showed that at this temperature the phosphorylating activity of the submitochondrial particles does not decrease for at least 40 min. Oxygen consumption was registered polarographically (PO-4 polarograph, Radiometer). The solubility of oxygen in the medium at 35° was

taken equal to 390 natoms/ml[17]. Whenever the reaction was started by introducing a suspension of particles preincubated with a substrate, the exact amount of oxygen in the polarographic cell was estimated using a platinum electrode calibrated to read oxygen content. Radioactivity was counted with a "Selo" counter. Analysis showed that the statistical deviation was not more than 3.5 % when counting 1000–2000 counts/min and 4.5 % when counting 40 000–50 000 counts/min. The amount of orthophosphate that passed into the form inextractable by an isobutanol–benzene mixture independently of oxidative phosphorylation was taken into account according to SCHATZ AND RACKER[15].

(2) *The rate of ATP-dependent NAD^+ reduction by succinate* was assayed after LÖW AND VALLIN[18]. The assay medium contained 37 mM Tris–HCl (pH 7.5), 2.2 mM $MgSO_4$, 10 mM succinate (pH 7.5), 180 mM sucrose, 1 mM KCN, 0.33 mg bovine serum albumin per ml, 0.7 mM NAD^+, 16 μg trypsin inhibitor per ml and submitochondrial particles (0.15 mg protein per ml). The reaction was initiated by introducing 1.3–2.0 mM ATP (pH 7.5). NAD^+ reduction was registered by the increase in absorbance of the solution at 340 nm (EPS-3 Hitachi or SP-700 Unicam spectrophotometer). The temperature during the measurements was 30°.

(3) *The rate of the ATP–$^{32}P_i$ exchange reaction* was assayed according to CHRISTIANSEN *et al.*[19].

(4) *The ATPase activity of particles* was assayed at 30° according to PULLMAN *et al.*[20]. The concentration of particles during the assays was 75 μg protein per ml. The concentration of phosphate liberated as a result of the ATPase reaction was estimated by the method of MARTIN AND DOTY[21]. In addition, this activity was assayed without using an ATP-regenerating system. In the latter case the medium contained 5 mM ATP (pH 7.5), 3 mM $MgCl_2$, 7.2 mM Tris–HCl (pH 7.4), 150 mM sucrose, 16 μg trypsin inhibitor per ml, and 10 mM glucose 6-phosphate. The latter was necessary to inhibit any hexokinase transferred together with the particles from the incubation medium to the assay medium. The reaction was initiated by adding 0.1 ml of the particle suspension (3.0 mg protein per ml) to 0.9 ml of the medium. After 10 min the reaction was interrupted by introducing 0.1 ml of 50 % trichloroacetic acid.

Conditions of incubation

The particles (3 mg protein per ml) were incubated at 30° with trypsin (15–20 μg/ml) in a medium containing 2 mM $MgCl_2$, 10 mM potassium phosphate (pH 7.5), 5 mM Tris–HCl (pH 7.5), 0.6 mM EDTA, 33 mM glucose, 120 mM sucrose and 0.25 mg hexokinase per ml. After the temperature was established (3–5 min) treatment of the particles was started under the conditions indicated in the legends to the figures. The volume of the incubation mixture was 2.0–3.0 ml. In the experiments with constant oxygen consumption incubation was carried out in Warburg vessels, shaking constantly, under conditions where oxygen diffusion was not a limiting factor.

RESULTS

Change in P/O ratio

It follows from Fig. 1 (Curve 1) that the phosphorylating ability of the submitochondrial particles, characterized by the P/O ratio, decreases rapidly in the presence of even small amounts of trypsin (0.006 mg/mg particle protein). Under the

same conditions the respiratory chain undergoes no perceptible changes. These observations are in agreement with the data of RACKER and co-workers[22,23,32], according to which, when trypsin acts on a phosphorylating system, the ATPase inhibitor and energy transfer factor F_3 are primarily destroyed.

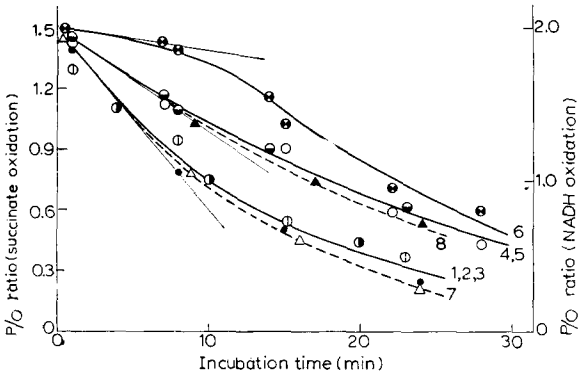

Fig. 1. Change in P/O ratio upon incubation of submitochondrial particles with trypsin. 1, control; 2, succinate 7 mM (pH 7.5), aerobic conditions; 3, ADP 2 mM (pH 7.5); 4, succinate *plus* ADP, trypsin added 3 min after these reagents; 5, particles were incubated with 2 mM ATP (pH 7.5) for 3 min, then trypsin was added; 6, particles were preincubated with ATP for 3 min, then hexokinase was added (0.25 mg/ml) and 2 min later, succinate and trypsin; 7, NADH-generating system (aerobic conditions); 8, NADH-generating system (30 mM ethanol, 0.2 mM NAD^+, 200 μg/ml alcohol dehydrogenase, and 6 mM semicarbazide) *plus* ADP, trypsin was added 3 min after the beginning of ADP phosphorylation. In all experiments represented on this and other figures the initial activities of the particles (A_0) were assayed immediately after introducing trypsin. The action of the latter was interrupted by transferring a particle suspension aliquot into the assay medium, which always contained soya trypsin inhibitor. The assays were made as described in MATERIALS AND METHODS. Solid lines, phosphorylation coupled to succinate oxidation was assayed. Broken lines correspond to phosphorylation coupled to NADH oxidation.

It was established in our previous papers that the respiratory chain (reconstituted from simple enzyme complexes or contained in submitochondrial particles) assumes a conformation resistant to proteolysis when NADH or succinate have been oxidized[9,10]. However, as is shown in Fig. 1 (Curves 2, 7) these substrates did not protect the phosphorylating system from the action of trypsin. Indeed, under aerobic conditions the P/O ratio decreased at the same rate, independent of whether the substrate was contained in the medium or not.

ADP itself did not stabilize the phosphorylating system either (Fig. 1, Curve 3). This result differs from the data according to which ADP is capable of controlling the structure of mitochondrial membranes[24,58]. A considerable decrease in the rate of inactivation of the phosphorylating system was observed only when PO_4^{3-}, ADP, and one of the substrates (NADH or succinate) were present simultaneously in the medium. The initial rate of inactivation decreased under the conditions indicated by a factor of two or more, and the half-inactivation time increased proportionally. Since the protective effect was independent of the nature of the substrate, it can hardly be attributed to the formation of specific triple complexes of the phosphorylating system with ADP and the substrate. This effect can hardly be associated either with ATP accumulation in the medium (ATP being capable of affecting mitochondrial structure substantially), since the particles were always incubated in the presence of

an ATP-trapping system*. Most probably, the increase in resistance to trypsin was due to the functioning of the phosphorylating system. This assumption will be discussed in greater detail in the following sections of this paper.

The maximal protective effect was observed when the particles were incubated for 2–3 min with ATP immediately before treatment with trypsin. After converting the excess ATP into ADP by means of the hexokinase reaction and adding succinate in aerobic conditions the particles retained an almost unchanged phosphorylating ability for some time in the presence of usual amounts of trypsin (Fig. 1, Curve 6). This effect was characteristic only of particles showing a P/O ratio of not under 1.5 with succinate used as the electron donor. Probably, the ability to be protected from proteolysis under conditions where oxidative phosphorylation is occurring is in direct dependence to the functional effectiveness of the phosphorylating system, *i.e.* to its intactness.

In contrast to ADP, ATP protected the phosphorylating system from proteolysis (see Fig. 1, Curve 5). Practically the same increase in the resistance of the particles to trypsin was observed when ATP was introduced into the medium together with trypsin in the absence of glucose and hexokinase as in the case where the particles were preincubated 2–3 min with ATP as has just been described above. The effect of ATP may be due either to a change in structure of the phosphorylating system as the result of the ATPase reaction or to its specific influence on this system through the formation of a stable complex[25]. The second assumption seems less likely, since ADP, which is an analog of ATP, did not protect the phosphorylating system from the action of trypsin.

The effect of ATP on the proteolysis of mitochondria was mentioned briefly by WEINBACH AND GARBUS[27].

Similar results were obtained while measuring other characteristics of the phosphorylating system: the rate of the ATP–$^{32}P_i$ exchange reaction and the rate of ATP-dependent NAD^+ reduction by succinate. A study of the effect of trypsin on the latter rate made it possible to establish some important details of the protecting effect which will be described in the next section.

Change in the rate of ATP-dependent NAD^+ reduction by succinate

To study the mechanism of stabilization of a phosphorylating system one must have information on the state of all three phosphorylation sites. This section gives data concerning Site I. A convenient criterion for assessing its state is the rate of ATP-dependent NAD^+ reduction by succinate. The rate of this reaction decreased when the particles were treated with trypsin (Fig. 2, Curve 1), which is evidence of the destruction of Site I. ATP synthesis coupled to oxidation of NADH or succinate is accompanied by substantial changes in resistance of the phosphorylating system to proteolysis. Results shown in Fig. 2 were obtained with NADH as the substrate. In all experiments a NADH-generating system was used, consisting of NAD^+, ethanol, alcohol dehydrogenase, and semicarbazide. It follows from Fig. 2 (Curve 2) that

* The experiment showed that under the conditions of incubation of the particles (see MATERIALS AND METHODS) v_{max} for hexokinase was about 10 μmoles of ATP per min per mg protein. The stationary ATP concentration calculated for this value of v_{max} and $K_m = 0.1$ (see ref. 26) did not exceed 0.05 mM. Taken in this concentration, ATP showed no protective action. Under the conditions indicated neither hexokinase nor alcohol dehydrogenase (contained in the NADH-generating system) were affected noticeably by trypsin in the course of 10–20 min.

NADH did not stabilize Site I in aerobic conditions. In a medium containing ADP and an incomplete NADH-generating system (without ethanol) inactivation of Site I due to its proteolysis was slowed down insignificantly (Curve 3, Fig. 2). The addition to this system of alcohol, which itself in no way influenced the resistance of the particles to trypsin, was accompanied by electron transfer, ADP phosphorylation, and simultaneously by distinct stabilization of the phosphorylating system (Curve 4, Fig. 2). In this case the stationary NADH concentration during incubation of the particles was very insignificant (see Fig. 2). The protective effect cannot be attributed to accumulation of ATP in the medium, since the latter always contained an ATP-trapping system (see above).

Results similar to those were obtained with succinate used as the substrate. In this case the phosphorylating system was the most resistant to proteolysis when succinate, ADP (and phosphate) were present in the medium simultaneously. After the succinate was exhausted in the incubation medium (which was determined polarographically), rapid inactivation of Site I set in. This inactivation could be retarded by adding a new portion of succinate. It follows from these results that proteolysis of Site I is slowed down under conditions where phosphorylation is coupled with electron transfer through the succinate oxidase system (*i.e.* when phosphorylation occurs in Sites II and III). This suggests that the structural organization of the phosphorylating system permits close interaction between the different phosphorylation sites.

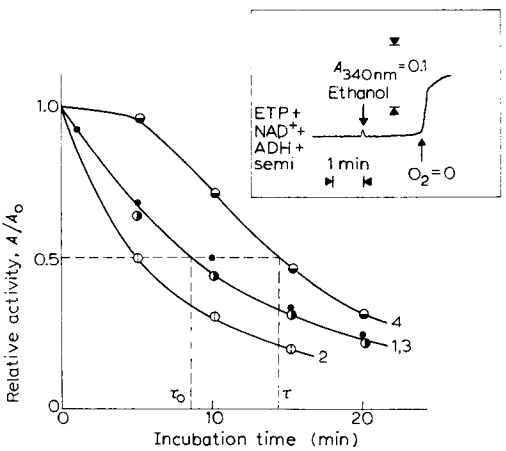

Fig. 2. Change in rate of ATP-dependent reduction of NAD^+ by succinate on incubation of particles with trypsin (incubation under conditions where ADP phosphorylation is coupled to NADH oxidation). 1, control ($A_0 = 81$); 2, complete NADH-generating system, aerobic conditions ($A_0 = 100$); 3, ADP *plus* NADH-generating system without ethanol ($A_0 = 65$); 4, complete NADH-generating system *plus* ADP ($A_0 = 95$). In all experiments trypsin was added 3 min after the other reagents. A_0 are expressed as nmoles NAD^+ reduced per min per mg particle protein. The rate of NADH oxidation under the conditions of incubation was 47 nmoles NADH per min per mg protein (Curves 2 and 4). Upper right-hand corner: change in concentration of NADH upon incubation of particles under conditions of Expt. 2. Assays were carried out as indicated in MATERIALS AND METHODS. To avoid errors due to the production of NADH by the generating system contained in the incubation medium one of two methods was used. (1) The particles were held in the assay medium for 4 min in the presence of 4 mM of semicarbazide to complete exhaustion of ethanol. Then KCN (no NADH was observed to form), succinate, and ATP were added. (2) The particles were introduced into an assay medium, which in addition to the components indicated contained 0.06 mM NADH and 4 mM semicarbazide. Under these conditions the NADH-generating system was completely suppressed. ETP = electron transport particles; ADH = alcohol dehydrogenase; semi = semicarbazide.

Important information on the relation between the functional state of an oxidative phosphorylation system and its resistance to proteolysis can be obtained by using agents which uncouple respiration and phosphorylation. Fig. 3 shows that the addition of sodium oleate to the incubation medium completely obliterated the stabilizing action of succinate and ADP described above. It was established preliminarily that 100 nmoles oleate per mg of particle protein completely suppressed ADP phosphorylation coupled with succinate oxidation, as well as ATP-dependent reduction of NAD^+ by succinate. Under the same conditions the rate of NADH oxidation by oxygen did not change. It follows from Fig. 3 that in the concentration indicated oleate itself did not increase the rate of proteolysis of the phosphorylating system (Fig. 3, Curve 2). This is an advantage of oleate over other uncoupling agents (2,4-dinitrophenol, pentachlorophenol, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) *etc.*), which increase the sensitivity of mitochondrial proteins to trypsin[27]. The latter is apt to complicate interpretation of experimental data. Another advantage of using oleate was that its action could be stopped completely by bovine serum albumin. To estimate the phosphorylating activity of particles incubated with trypsin, oleate and other components, it was sufficient to add 1 mg bovine serum albumin per ml to the assay medium. This eliminated artifacts which might have appeared with more complex procedures of uncoupler removal.

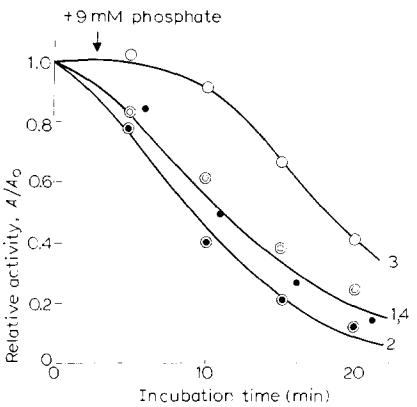

Fig. 3. Effect of oleate on protective action of succinate and ADP upon treatment of particles with trypsin (changes in the rate of ATP-dependent reduction of NAD^+ by succinate). 1, control ($A_0 = 160$); 2, oleate (100 nmoles/mg of particle protein) ($A_0 = 145$); 3, 15 mM succinate *plus* 2 mM ADP ($A_0 = 216$); 4, oleate *plus* succinate *plus* ADP, reagents were introduced in the sequence indicated ($A_0 = 200$). In all experiments trypsin was added 6 min after succinate and ADP. To remove the trace amount of ATP, ADP was preincubated with hexokinase 10 min before particles and succinate were introduced into incubation medium. The latter contained 10 mM potassium phosphate (pH 7.5), 20 mM Tris–HCl (pH 7.5), 120 mM sucrose, 32 mM glucose, 4 mM $MgSO_4$, 0.5 mM EDTA, particles (3 mg protein per ml) and 7.2 μg trypsin per mg particle protein. Assay medium was as described in MATERIALS AND METHODS except that amount of bovine serum albumin was 1 mg/ml. A_0 is expressed as nmoles NAD^+ reduced per min per mg protein.

ATP hydrolysis and ADP phosphorylation result in opposite changes in the concentration of H^+ in the medium[28]. However, as was proved by special experiments when the particles were incubated with ATP or ADP in the presence of the substrate, pH alteration which did not exceed ± 0.1 cannot account for the protective effect.

Optimal conditions for stabilization of a phosphorylating system

Fig. 4A shows how the relative increase in half-inactivation time of the phosphorylating system by trypsin $(\tau-\tau_0)/\tau_0$ changes with changing rate of electron transfer (see, for instance, Fig. 2). This dimensionless value can be used for a quantitative estimate of the stabilization effect. The rate of electron transfer was varied by changing only the content of alcohol dehydrogenase in the incubation medium. The concentrations of all the other components of the medium, including ADP, NAD^+, ethanol, and semicarbazide, were the same in all experiments. It follows from Fig. 4A that at low rates of NADH oxidation the phosphorylating system was not protected from the action of trypsin. With the increasing rate of electron transfer there was a sharp increase in the resistance of the system to proteolysis. Further acceleration of electron transfer was accompanied by just as sharp destabilization of the system. At fairly high rates of NADH oxidation no stabilization occurred at all. It should be emphasized that assays of different characteristics of the phosphorylating system (P/O ratio and rate of ATP-dependent reduction of NAD^+ by succinate) gave coinciding results.

To ascertain the nature of this effect it was necessary to verify how the effectiveness of phosphorylation changes with the changing rate of NADH oxidation under the experimental conditions indicated. We proved that at low rates of electron transfer the effectiveness of phosphorylation was high (Fig. 4B). Increasing the rate of this process lowered the P/O ratio to 1, after which this ratio remained unchanged. Comparison of Figs. 4A and 4B shows that stabilization of the phosphorylating system occurs only when ADP phosphorylation proceeds sufficiently rapidly and effectively. An excessive increase in the rate of electron transfer causes substantial changes in

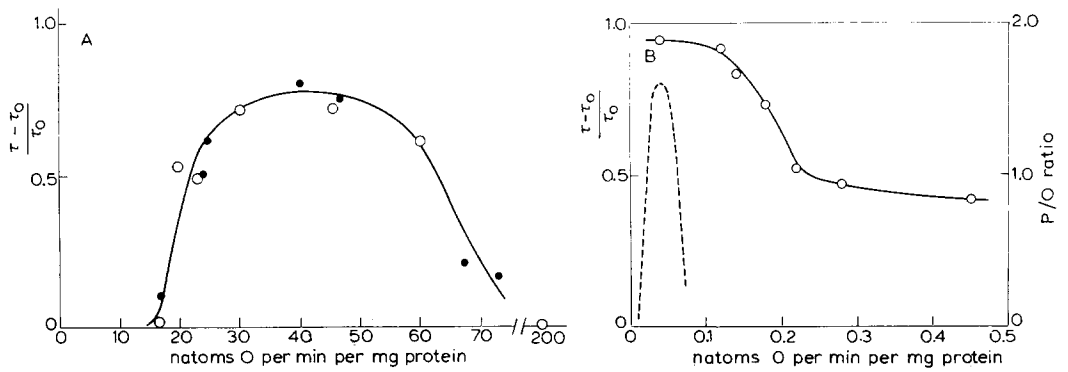

Fig. 4. A. Stabilization of phosphorylating system at various rates of electron transfer through NADH oxidase chain in the presence of ADP. The conditions of incubation of the particles were as in Fig. 2. The rate of NADH oxidation was regulated by varying the concentration of alcohol dehydrogenase from 50 to 700 μg/ml. This rate was determined polarographically while incubating the particles. τ_0 is the half-inactivation time of the phosphorylating system in the control (see, for example, Fig. 2), τ is the half-inactivation time of the system upon incubation of the particles in the presence of a complete NADH-generating system and ADP, $(\tau-\tau_0)/\tau_0$ is the relative increase in half-inactivation time. ○, rate of ATP-dependent reduction of NAD^+ by succinate was assayed; ●, P/O ratio was assayed (phosphorylation was coupled to NADH oxidation). B. Dependence of effectiveness of phosphorylation on rate of NADH oxidation. Rate of NADH oxidation was regulated by varying the concentration of alcohol dehydrogenase from 40 to 400 μg/ml. Concentration of particles during assays was 0.9 mg/ml. Assays were carried out as indicated in MATERIALS AND METHODS. The broken curve represents data given in A.

the oxidative phosphorylation system, manifested first as a disappearance of the protective effect and then as a lowering of its phosphorylating ability. The decrease in the P/O ratio in our experiments was not connected with the uncoupling action of NADH[29], since the stationary concentration of the latter was very insignificant (see Fig. 2).

SCHATZ AND RACKER[15] have shown previously that at high NADH oxidation rates the effectiveness of phosphorylation decreases. On the other hand, the P/O ratio does not change over a wide range of succinate oxidation rates[30]. In connection with this, it is interesting to note that the stabilizing action of succinate in the presence of ADP did not disappear at high rates of electron transfer (about 200 natoms of oxygen per min per mg protein).

Change in ATPase activity

It follows from Fig. 5 that treatment of the particles with trypsin increases their ATPase activity approximately 2-fold. According to the data of RACKER and co-workers[22,31,32], this effect is due to the destruction of the ATPase inhibitor. The specific feature of ATPase activation induced by trypsin is that neither ATP nor ADP in the presence of succinate affect this process (Fig. 5). It was shown by HORSTMAN AND RACKER[33] that in the presence of Mg^{2+} ATP influences the interaction of the inhibitor both with soluble ATPase, and with the enzyme linked to the particles. It follows from Fig. 5 that even if ATP promotes strengthening of the bond between ATPase and its inhibitor, it does not change the resistance of the latter (in the particles) to trypsin. The results of this section suggest that the changes induced in the phosphorylating system by ATP or ADP in the presence of succinate do not substantially affect the region where the ATPase inhibitor is localized.

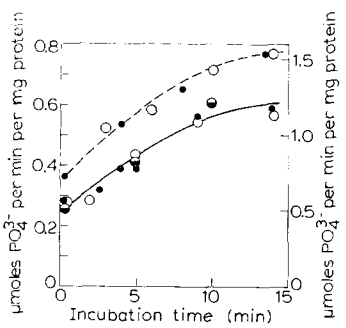

Fig. 5. Change in ATPase activity during incubation of particles with trypsin. ●, control; ◒, incubation with 2.0 mM ATP; ○, 7.0 mM succinate *plus* 2.0 mM ADP, aerobic conditions. The broken line corresponds to the experiments where activities were assayed in the presence of an ATP-generating system (activity values are shown at the right). In assays of the activities of particles incubated with succinate 0.03 mM of thenoyltrifluoroacetone was added to the medium. The activities are expressed in μmoles P_i evolved per min per mg of particle protein.

DISCUSSION

Some time ago there was an extensive discussion as to how the swelling and shrinking of mitochondria is related to oxidative phosphorylation. It was assumed at first that oxidative phosphorylation or a fairly high level of intramitochondrial

ATP are necessary to maintain the particles in a compact form[34–43]. Since the swelling of mitochondria under certain conditions is a result of aging, the data given in the papers mentioned above can be regarded as the first indication of the possible dependence of the rate of aging on the functional state of the mitochondria. However, there is no uniform opinion on this question. According to the data of a number of authors, oxidative phosphorylation is not necessary to prevent swelling of mitochondria[24, 44–48].

Evidently solution of this problem requires a more detailed study of the nature of the factors causing aging of mitochondria and of the factors retarding this process. In recent years experimental data have been appearing, according to which aging of mitochondria *in vitro* and their degradation *in vivo* are related to acceleration of proteolysis and splitting of mitochondrial phospholipides[1–7]. In the light of these data the protection of mitochondria from the action of lytic enzymes becomes a problem of special importance. It seems necessary first of all to establish the principles of stabilization of the oxidative phosphorylation system owing to the key importance of this system for mitochondria.

Results for the respiratory chain and a number of simple enzyme complexes contained in it were published previously by us in a series of papers[8–12]. According to these results, in the presence of oxidizable substrates and oxygen (*i.e.* under conditions favouring electron transfer) the resistance of the respiratory chain to proteolysis and the action of cobra venom phospholipase (*i.e.* phospholipase A) increases.

In this work we studied the action of trypsin on the phosphorylating system of submitochondrial particles. The main conclusion is that the state of the phosphorylating system wherein it is resistant to trypsin arises when it is functioning. This conclusion was drawn from the following experimental facts: (1) electron transfer is not accompanied by retarding inactivation of the phosphorylating system by trypsin, (2) in the presence of phosphate ADP does not stabilize this system either, (3) proteolysis is retarded when particles are transferring electrons in a medium containing ADP (phosphate acceptor) and phosphate, (4) when the oxidizable substrate is exhausted the phosphorylating system becomes destabilized, (5) the stabilizing action of succinate and ADP vanishes when oleate is added in the concentrations necessary for complete uncoupling of respiration and phosphorylation, (6) the protective effect depends on the rate and effectivity of ADP phosphorylation (in the case of NADH oxidation).

It is known that a change in the rate of proteolysis of proteins (including mitochondrial proteins[49, 50]) is evidence of a change in their structure. For this reason the results of this work may be regarded as proof of structural changes in the phosphorylating system of submitochondrial particles.

According to numerous experimental data obtained in recent years (electron microscopy data[51, 52], the results of light scattering measurements[52, 53], 8-anilinonaphthalene-1-sulfonate fluorescence[54, 55], optical rotatory dispersion[56], *etc.*) the structure of mitochondrial membranes, and hence their components, depends on the functional state of the oxidative phosphorylation system. In the papers mentioned above it was assumed that oxidizable substrates and ATP cause identical structural changes in submitochondrial particles. However, as was shown above, electron transfer itself does not affect the structure of the phosphorylating system, or at least that part of

it which is sensitive to trypsin. The structure of this system undergoes changes only under conditions where ADP is being phosphorylated or ATP is being hydrolyzed. At the same time electron transfer is accompanied by an increase in the thermostability of the respiratory chain and its resistance to the action of lytic enzymes, which is due most probably to structural changes in this system[8–12]. Possibly, the differences indicated between the results of this and a number of previous papers[52–56, 59] are due to the fact that with the methods used earlier the conformational changes in the different components of the mitochondrial membranes could not be distinguished.

RACKER and co-workers[22, 23, 31, 32] showed that while acting on a phosphorylating system trypsin primarily splits the coupling factor F_3 and the ATPase inhibitor. Stabilization of this system probably consists in a decrease in access of the F_3 factor to the trypsin molecules. At the same time the conformational changes in the phosphorylating system of submitochondrial particles are not affected substantially by the sites of localization of the ATPase inhibitor. Apparently the phosphorylating system components mentioned are sufficiently remote from one another in the crystal membrane.

It follows from this work that the oxidative phosphorylation system is stable against proteolysis only in a medium where a definite concentration of NADH, succinate, ADP, and phosphate is maintained. This condition is fulfilled in intact mitochondria, which contain numerous auxiliary enzymes supplying substrates to the respiratory chain, as well as membranes preventing the leakage of substrates, cofactors and soluble proteins into the surrounding medium[37–39, 57, 58]. In this connection it seems quite likely that any influences on the mitochondria and any deviations in their composition which are accompanied by a disturbance of the functions of the oxidative phosphorylation system should result in destabilization of the latter, manifested in particular by an increase in its thermal lability[12] and its sensitivity to proteolysis. This assumption, if it is true, may help in the search for the principles controlling the assembly of mitochondria.

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