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STUDIES ON STABILIZATION OF THE OXIDATIVE PHOSPHORYLATION SYSTEM

II. ELECTRON TRANSFER-DEPENDENT RESISTANCE OF SUCCINATE OXIDASE AND NADH OXIDASE SYSTEMS OF SUBMITOCHONDRIAL PARTICLES TO PROTEINASES AND COBRA VENOM PHOSPHOLIPASE

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SUMMARY

Inactivation of the NADH oxidase system of submitochondrial particles by trypsin (or chymotrypsin) and inactivation of the succinate oxidase system by cobra venom phospholipase has been studied. The following results were obtained:

1. Inactivation of NADH oxidase slows down in the presence of NADH and oxygen. The protective effect decreases or disappears completely when compounds hindering electron transfer (Zn^{2+}, NAD^+, deamino NAD^+ and thionicotinamide NADH) are added to the incubation medium. The mentioned inhibitors *per se* do not increase the sensitivity of NADH oxidase to proteolytic enzymes.

2. Similar results are obtained when deamino NADH is used as a substrate. In this case the protective effect also decreases in the presence of Zn^{2+} and deamino NAD^+.

3. The NADH analogues, unable to supply electrons for NADH oxidase, do not protect it from the action of proteinases. This refers to NADPH, thionicotinamide NADH, their oxidized forms, and also NAD^+ and deamino NAD^+.

4. Succinate retards inactivation of the succinate oxidase by cobra venom phospholipase. Zn^{2+} and malonate interfere with electron transfer and reduce the protective effect of succinate.

5. Succinate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) taken together under anaerobic conditions do not increase the resistance of succinate oxidase to the action of cobra venom phospholipase. Reduced TMPD in the presence of oxygen does not stabilize the system either.

6. Inactivation of cytochrome oxidase in the submitochondrial particles by phospholipase is not retarded in the presence of the reduced TMPD and oxygen. Some stabilization of this fragment occurs in the presence of succinate.

A conclusion is made that the rate of electron transfer is one of the factors determining the life-time of the respiratory chain in the presence of proteinases, as well as cobra venom phospholipase. Stabilization is possible only when electrons are transferred across all the labile sites of the multi-enzyme system.

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

INTRODUCTION

Several years ago it was established that the NADH oxidase and succinate oxidase systems acquire resistance to cobra venom phospholipase and proteinases (trypsin or chymotrypsin) when NADH or succinate are present in the medium. The protective action of the substrates revealed itself only in aerobic conditions[1,2]. In other words, unlike the functioning form of the respiratory chain, neither its reduced nor oxidized forms were resistant to lytic enzymes. This gave grounds to believe that when the electrons are transferred across the respiratory chain, it acquires a specific stable conformation. The alternative suggestion that stabilization is connected with the formation of an enzyme–substrate complex could not be ruled out, however.

This work is a further study of the protective effect. It proves that electron transfer plays the primary role in the stabilization of the NADH oxidase system. Results have been obtained according to which, NADH analogues, incapable of supplying electrons for this system, do not protect it from the action of chymotrypsin or trypsin even in the case where they are associated effectively with NADH oxidase. It was also established that the protective effect depends, all other conditions being equal, on the rate of electron transfer.

Identical results were obtained when studying the inactivation of the succinate oxidase system by cobra venom phospholipase. It has been established that the life-time of succinate oxidase in the presence of cobra venom depends on the rate of electron transfer. Electron transfer across the whole system is required to stabilize succinate oxidase. Functioning of some of its fragments does not prevent either these fragments or the multi-enzyme system from being disintegrated.

MATERIALS AND METHODS

Submitochondrial particles

Ultrasonic submitochondrial particles isolated from beef heart mitochondria by the method of Beyer[3] or alkaline submitochondrial particles obtained according to the method Crane *et al.*[4] were used.

Enzyme assays

The NADH oxidase. The activity was assayed at 30 °C or 38 °C. A decrease in the absorbance of the solution was recorded at 340 nm. The extinction coefficient of NADH was taken to be equal to 6.22 $mM^{-1} \cdot cm^{-1}$. The assay medium contained 80 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA and 0.1 mM NADH. The reaction was initiated by addition of the particles (the final concn was 3–20 μg protein per ml. NADH was the last to be introduced in the cases when particles were incubated with trypsin or chymotrypsin actually in the cuvette of a spectrophotometer. When the proteolysis of particles was carried out in the presence of Zn^{2+}, the inhibitory action of the latter on NADH oxidase was completely prevented by adding EDTA to the assay medium (final concn 2 mM). The incubation mixture was diluted 1:50 or 1:80 by the assay medium to eliminate the inhibitory effect of NADH analogues.

Succinate oxidase. The activity was assayed at 38 °C by recording the increase

in the absorbance of the solution at 230 nm. Extinction coefficient of fumarate was assumed to be 4.38 $mM^{-1} \cdot cm^{-1}$. The assay medium contained 80 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA and 20 mM succinate. As succinate oxidase may be activated by phosphate and succinate, maximum activity was estimated in all experiments after 10 min preincubation of the particles in the medium of the above composition. The reaction was initiated by addition of the particles (the final concentration 20 μg protein per ml). When the particles were preincubated in the medium containing *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), the assay medium contained 175 μM ascorbate which prevented electron transfer from succinate to TMPD. The incubation medium was diluted 1:50 by the assay medium to eliminate the inhibitory effect of malonate.

Cytochrome oxidase. The activity was assayed at 38°C. Oxidation of reduced TMPD was followed, which was accompanied by an increase in the absorbance of the solution at 562 nm. The assay medium contained, besides potassium phosphate buffer and EDTA, 10 mM TMPD. The reaction was initiated by adding the particles (20 μg protein per ml).

All the measurements were carried out with Hitachi EPS-3, Cary 15 or Unicam SP-700 recording spectrophotometers.

Analysis of the steady-state of the succinate oxidase and NADH oxidase systems

Steady-state levels of reduced cytochromes *b*, $c_1(+c)$ and aa_3 were determined by measuring differences in absorbance at 562–575 nm, 550–540 nm and 605–630 nm, respectively. Measurements were carried out with a Hitachi-356 dual wavelength spectrophotometer.

Incubation of submitochondrial particles

Treatment of submitochondrial particles with proteolytic enzymes was carried out actually in the cuvette of a spectrophotometer or in test tubes. In this case the incubation medium consisted of 80 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA and other components, indicated in the legends to the figures. The medium did not contain EDTA when NADH oxidase was inhibited by Zn^{2+}. The stabilizing effect of substrates was observed under aerobic conditions in the presence of NADH or deamino NADH. Aerobic conditions were created by shaking the particle suspension (250–500 μg protein per ml) in Warburg vessels. The electron donor concentration was kept approximately constant during the incubation of particles. Small amounts of substrate were added from time to time and its concentration was not allowed to drop more than 20 % from the initial level.

In experiments with cobra venom the incubation medium consisted only of 80 mM potassium phosphate buffer (pH 7.4) and additives, indicated in the legends to the figures.

Reagents

The lyophilized cobra venom used was prepared in the Institute of Zoology and Parasitology of the Academy of Sciences of the Uzbek SSR (Tashkent). As was shown by Edwards and Ball[5], cobra venom disintegrates the respiratory system exclusively due to the action of the phospholipase A it contains. Indeed, other enzymes present in cobra venom[6] are not likely to cause inactivation of the respiratory

system. In our experiments the inactivating effect of cobra venom could be completely eliminated by 1 mM EDTA, which agrees with what is known of purified phospholipase A (see ref. 7)*.

NAD+, NADH, NADP+, NADPH and thionicotinamide NAD+ were purchased from Boehringer Mannheim. Deamino NAD+ was obtained from Sigma Chemical Co. Thionicotinamide NAD+ and deamino NAD+ were reduced according to the method of Minakami *et al.*[10]. TMPD was twice crystallized.

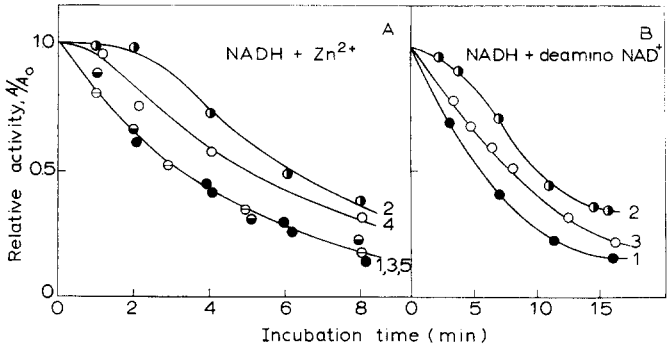

Fig. 1. Effects of Zn^{2+} and deamino NAD+ on the stabilization of the NADH oxidase system by NADH. A. The alkaline submitochondrial particles were incubated with chymotrypsin in the cuvette of a spectrophotometer at 30 °C. The concentration of particles during incubation was 20 μg protein per ml; chymotrypsin concn, 8.0 mg/ml. EDTA was added before the measurements of the NADH oxidase activity (final concn 2 mM). The other incubation conditions are indicated below. 1, incubation of particles without NADH and Zn^{2+}; 2, in the presence of 0.1 mM NADH (aerobic conditions); 3, in the presence of 0.2 mM Zn^{2+}; 4, in the presence of 20 μM Zn^{2+} and 0.1 mM NADH (40% inhibition of NADH oxidase); 5, in the presence of 0.2 mM Zn^{2+} and 0.1 mM NADH (64% inhibition). The inhibition was estimated under the conditions of particle incubation. A_0, assayed at 30 °C immediately after introducing chymotrypsin amounted to 0.7 μmole NADH per min per mg protein. B. The alkaline submitochondrial particles were incubated with trypsin at 30 °C. The concentration of particles was 250 μg protein per ml; trypsin concn, 40 mg/ml. The other incubation conditions are given below. Aliquots of particle suspension were withdrawn after definite time intervals and the NADH oxidase activity was assayed at 30 °C. The concentration of particles during assay was 10 μg protein per ml (dilution of incubation medium 1:25). A_0, assayed immediately after introducing trypsin, amounted to 1.3 μmoles NADH oxidized per mg protein per min. 1, incubation without NADH and deamino NAD+; 2, in the presence of 1.15 mM NADH (aerobic conditions); 3, in the presence of 1.15 mM NADH and 23.0 mM deamino NAD+ (30% inhibition of NADH oxidase). The inhibition of NADH oxidase was assayed when the concentrations of particles, NADH and deamino NAD+ were 1:10 in respect to the ones used under the conditions of particle incubation.

RESULTS

Dependence of the resistance of NADH oxidase to proteinases on the rate of electron transfer

Various inhibitors of the NADH oxidase system were used to solve the problem of how the protective effect of substrates changes with the alteration of the rate of electron transfer. One of them (Zn^{2+}) hindered electron transfer mainly at the site

* Some authors[5,8,9] used cobra venom as a crude preparation of phospholipase A which was heated to eliminate concomitant enzymatic activities. In our experiments preheating of cobra venom for 10 min at 95 °C (pH 5.8) did not markedly decrease its effect on the succinate oxidase system. This also suggests that the inactivation of the latter was due to the action of phospholipase A.

between cytochromes *b* and c_1 (ref. 11), while the others (NAD^+, deamino NAD^+ and thionicotinamide NADH) competed with the substrate for the active centre of the enzyme*. In all the cases the action of the inhibitors was prevented by various methods before NADH oxidase activity had been assayed (see Materials and methods).

As seen in Fig. 1A, NADH retards the inactivation of the NADH oxidase system by chymotrypsin. Addition of Zn^{2+}, besides NADH, to the incubation medium brings about a decrease of the protective effect. The latter disappears completely when the rate of electron transfer diminishes by about 70 %. Addition of Zn^{2+} (20 μM) does not alter the affinity of the substrate (NADH) for the enzyme. This follows from a comparison of K_m values found for NADH oxidase in the presence and absence of Zn^{2+} (in the both cases K_m was 4.7 μM for ultrasonic submitochondrial particles). It ought to be stressed that Zn^{2+} *per se* produces no influence on the resistance of NADH oxidase to chymotrypsin. Therefore, the decrease of the protective effect should be ascribed only to the retarding of the electron flow.

TABLE I

CHANGES IN THE STEADY-STATE LEVELS OF REDUCED CYTOCHROMES INDUCED BY Zn^{2+} AND DEAMINO NAD^+

The concentration of alkaline submitochondrial particles during measurements was 0.5 mg protein per ml, concentration of NADH was 1.4 mM. Temp. 32 °C.

Substrate	*Inhibitor*	*Concn of inhibitor (mM)*	*Inhibition** (%)	*Steady-state levels of reduced cytochromes*		
				Cytochrome aa_3	*Cytochrome c_1 (+ c)*	*Cytochrome b*
NADH				18	43	47
NADH	Zn^{2+}	0.04	75	12	26	61
NADH				15	40	61
NADH	Deamino NAD^+	56.0	50	24	46	51

* Inhibition of NADH oxidase was assayed by the time of oxygen exhaustion in the cuvette of a Hitachi 356 spectrophotometer during analysis of the steady state of the system.

As seen from Table I, the steady-state levels of reduced cytochromes change substantially during oxidation of NADH in the presence of Zn^{2+}. In this case the content of the reduced form of cytochrome *b* increases and the content of the reduced forms of cytochromes $c_1(+c)$ and aa_3 decreases.

Somewhat different results were obtained when using competitive inhibitors of NADH oxidase: NAD^+, deamino NAD^+, and thionicotinamide NADH**. These

* According to our data plots of $1/v$ against $1/[S]$ are linear and the lines for various inhibitor (deamino NAD^+ or thionicotinamide NAD^+) concentrations meet the $1/v$ axis at a constant intercept. This suggests a competitive character of NADH oxidase inhibition by the mentioned compounds.

** In these experiments the inhibition of NADH oxidase under the conditions of the incubation of the particles did not exceed 50%. A more effective deceleration of electron transfer required high concentrations of NADH analogues. This proved impossible because it was difficult to prevent the inhibitory action of analogues prior to the assay of NADH oxidase activity.

compounds also eliminated the protective effect of NADH and did not influence the rate of the proteolysis of the NADH oxidase. However, as distinct from Zn^{2+}, they did not cause dramatic changes in the steady-state levels of reduced cytochromes (Table I). Fig. 1B gives the results of the experiments in which the particles were incubated with trypsin in the presence of NADH and deamino NAD^+. Experiments with NADH analogues show that to reduce the protective effect, a decrease, all other conditions being equal, in the rate of electron transfer is apparently sufficient (see Figs 1A and 1B, and Table I).

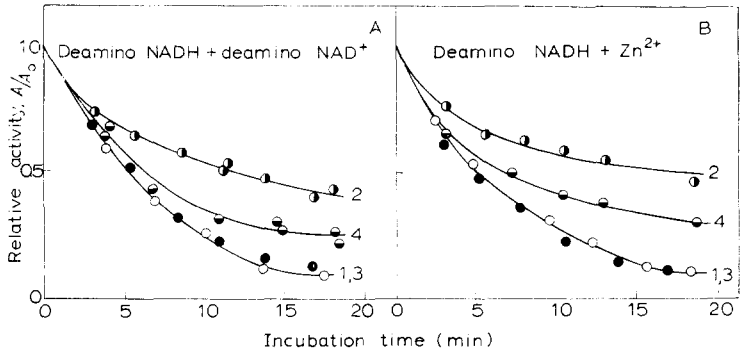

Fig. 2. Effects of Zn^{2+} and deamino NAD^+ on the stabilization of the NADH oxidase system by deamino NADH. The alkaline submitochondrial particles were incubated with trypsin at 32 °C. The concentration of particles was 250 μg protein per ml, trypsin concn, 40 mg/ml. The other incubation conditions are given below. A_0, assayed in the presence of trypsin at 38 °C, amounted to 2.3 μmoles NADH oxidized per min per mg protein. A. 1, incubation without deamino NADH and deamino NAD^+; 2, in the presence of 1.15 mM deamino NADH (aerobic conditions); 3, in the presence of 69 mM deamino NAD^+; 4, in the presence of 1.15 mM deamino NADH and 69 mM deamino NAD^+ (50% inhibition of deamino NADH oxidase activity). The inhibition was assayed under the conditions when the concentrations of particles, deamino NADH and deamino NAD^+ were all 1:10 in respect to the ones used under the conditions of particle incubation. The concentration of particles during assay was 3 μg protein per ml. B. 1, incubation without deamino NADH and Zn^{2+}; 2, in the presence of 1.15 mM deamino NADH (aerobic conditions); 3, in the presence of 0.1 mM Zn^{2+}; 4, in the presence of 1.15 mM deamino NADH and 0.1 mM Zn^{2+} (50% inhibition of deamino NADH oxidase activity). The inhibition was assayed under the conditions of particle incubation.

Deamino NADH, which in the presence of particles oxidized at a rate close to that of NADH oxidation, also protected the NADH oxidase system from proteolysis (Fig. 2). True, the protective effect in this case differed somewhat from that of NADH (*cf.* Figs 1 and 2). The reason for this difference is not clear. The protective effect of deamino NADH diminished when deamino NAD^+ or Zn^{2+} were added to the incubation medium. It is very interesting that both inhibitors, despite the difference in the mechanism of their action, decreased identically the protective effect when taken in amounts necessary to suppress electron transfer to the same level (Fig. 2). The same conclusion may be drawn from a comparison of Figs 1A and 1B.

Effect of NADH analogues on the resistance of the NADH oxidase system to proteolysis

According to Minakami *et al.*[10] NADH analogues are capable of associating effectively with soluble NADH dehydrogenase. These data as well as the results obtained by us for NADH oxidase of submitochondrial particles are listed in Table II.

Our experiments have shown that NADPH and thionicotinamide NADH, as well as their oxidized forms, do not protect NADH oxidase from the action of trypsin and chymotrypsin even in the case when their concentrations exceed the concentration

TABLE II

EFFECTS OF NADH AND ITS ANALOGUES ON THE PROTEOLYSIS OF THE NADH OXIDASE SYSTEM OF ALKALINE SUBMITOCHONDRIAL PARTICLES

The data for NADH oxidase were obtained under the following conditions: 80 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 1.55 μg particle protein per ml, temp. 30 °C. (+) indicates the presence, (−) the absence and (+) (−) the decrease of the protective effect.

Incubation conditions					*K_m for NADH dehydrogenase** (μM)	*K_m for NADH oxidase* (μM)
Compound (substrate, analogue, inhibitor, or their combination)	*Concn* (*mM*)	*V* (%)	*Inhibition* (%)	*Protective effect*		
NADH	0.065	100		(+)	108	11
Deamino NADH	0.080	100		(+)	40	20
NADPH	0.48	0		(−)		
Thionicotinamide NADH	0.16	1		(−)	24	4
NAD^+	2.0	0		(−)		
Deamino NAD^+	0.64	0		(−)		
$NADP^+$	0.23	0		(−)		
Thionicotinamide NAD^+	0.16	0		(−)		
NADH + Zn^{2+}	0.1 + 0.2		64	(−)		
NADH + deamino NAD^+	1.15 + 23		30	(+) (−)		
NADH + thionicotinamide NADH	0.065 + 0.1		50	(+) (−)		
Deamino NADH + Zn^{2+}	0.1 + 0.1		50	(+) (−)		
Deamino NADH + deamino NAD	1.15 + 69		56	(+) (−)		

* According to Minakami *et al.* (see ref. 10).

TABLE III

EFFECT OF Zn^{2+} AND MALONATE ON STABILIZATION OF THE SUCCINATE OXIDASE SYSTEM BY SUCCINATE. CHANGES IN THE STEADY-STATE LEVELS OF REDUCED CYTOCHROMES INDUCED BY Zn^{2+} AND MALONATE

Ultrasonic submitochondrial particles (0.83 mg protein per ml) were incubated with cobra venom (2.5 mg/ml) and other components indicated at 18 °C. The steady-state levels of reduced cytochromes were measured at 22 °C. In this case the medium contained 80 mM potassium phosphate buffer (pH 7.4), 0.6 mg particle protein per ml and, when necessary, zinc acetate or malonate. The reaction was initiated by adding succinate (final concn 20 mM).

Incubation conditions				*Steady-state levels of reduced cytochromes**		
Additives (substrate or substrate plus inhibitor)	*Concn* (*mM*)	*Inhibition* (%)	*Protective effect*	*Cytochrome aa_3*	*Cytochrome c_1 (+ c)*	*Cytochrome b*
Succinate	20		(+)	9	20	35
Succinate + Zn^{2+}	20 + 0.04	23	(+) (−)	9	14	48
Succinate	20		(+)	14	22	25
Succinate + malonate	20 + 0.5	50	(+) (−)			
Succinate + malonate	20 + 1.0	70	(−)	13	21	23

* As percentage of that when completely reduced by succinate.

of NADH (see Table II). It follows from Table II that formation of a specific complex with the substrate or its analogue cannot be the reason for increased resistance of the NADH oxidase system to proteolysis. Indeed, if the K_m values for NADH, deamino NADH and thionicotinamide NADH are compared it is easily seen that the latter associates best with NADH dehydrogenase (or NADH oxidase). In its presence, however, inactivation of NADH oxidase by chymotrypsin or trypsin dit not retard at all. The protective action is characteristic only of compounds that are capable of supplying electrons for the NADH oxidase system.

Resistance of succinate oxidase to cobra venom in the case of electron transfer across the respiratory chain fragments

Results were obtained similar to the ones described above for the NADH oxidase system when the succinate oxidase of submitochondrial particles was subjected to the action of cobra venom phospholipase. Inactivation of succinate oxidase was retarded in the presence of succinate (aerobic conditions). Zn^{2+} (noncompetitive inhibitor) and malonate (competitive inhibitor) interfering with electron transfer, reduced or completely eliminated the protective effect of succinate (Table III). By themselves these inhibitors did not accelerate the inactivation of succinate oxidase by cobra venom. Table III gives also data related to the influence of malonate and Zn^{2+} on the steady-state levels of reduced cytochromes.

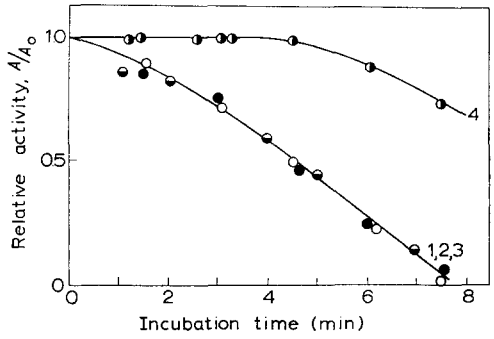

Fig. 3. Resistance of succinate oxidase system to cobra venom in the case of electron transfer across limited sections of respiratory chain. The ultrasonic submitochondrial particles (0.83 mg protein per ml) were incubated with cobra venom (2.5 mg/ml) at 18 °C in the medium containing 80 mM potassium phosphate buffer (pH 7.4). At the time intervals indicated 0.05-ml aliquots of particle suspension were taken and their succinate oxidase activity was measured as described in Materials and Methods. A_0, assayed immediately after introducing cobra venom, amounted to 1.4 μmoles succinate per min per mg protein. 1, incubation without succinate and TMPD; 2, in the presence of 20 mM succinate and 1 mM TMPD, anaerobic conditions; 3, in the presence of 0.1 mM TMPD and 1 mM ascorbate, aerobic conditions; 4, in the presence of 20 mM succinate, aerobic conditions.

In all the mentioned experiments electron transfer during the incubation of particles with cobra venom was effected through the entire succinate oxidase system. The question arises whether stabilization is possible when electrons are transferred across the respiratory chain fragments. It can be answered by using artificial electron donors and acceptors. As was demonstrated above, the protective effect depends upon the rate of electron transfer. Hence one should employ compounds which are

oxidized or reduced by the respiratory chain at a sufficiently high rate. TMPD meets these requirements. On being oxidized TMPD accepts electrons at the levels of flavoprotein F_s and cytochrome *b* (ref. 13). Reduced TMPD donates electrons to cytochrome c_1 (refs 13 and 14). Both processes, under certain conditions, show a rate close to that of succinate oxidation by oxygen in the presence of submitochondrial particles.

Fig. 3 shows that when succinate and TMPD are added to the particles incubated under anaerobic conditions, no increase in the resistance of succinate oxidase system to cobra venom phospholipase is observed. TMPD in the presence of ascorbate (aerobic conditions) does not decrease the rate of inactivation of this system either (Fig. 3). Initial rates of oxidation of succinate and reduced TMPD during incubation of particles with cobra venom were 150 and 190 nmoles/min per mg protein, respectively, which are close to succinate oxidase activity of the particles (160 nmoles succinate/min per mg protein). TMPD *per se* does not affect the resistance of the particles to cobra venom (Fig. 3).

Thus, electron transfer across the respiratory chain fragments does not entail its stabilization. What happened to these fragments then? It was demonstrated previously[1] that the rate of inactivation of isolated NADH:cytochrome *c* oxidoreductase (Complex I + III) by cobra venom phospholipase does not decrease in the presence of cytochrome *c* and NADH. Fig. 4 shows similar results for cytochrome oxidase linked to submitochondrial particles. One sees that cytochrome oxidase activity of the particles, measured by the rate of reduced TMPD oxidation, decreases when cobra venom is added to the particles. Succinate retards inactivation under aerobic conditions, whereas electron transfer from TMPD *via* cytochromes c_1 and *c* and cytochrome oxidase to oxygen is not accompanied with such an effect.

DISCUSSION

The main conclusion of this work is that electron transfer is necessary to increase the resistance of the respiratory chain to lytic enzymes (trypsin, chymotrypsin and cobra venom phospholipase). This conclusion can be supported by three most important facts: (1) to protect the respiratory chain it is necessary to have both substrate (NADH, deamino NADH or succinate) and oxygen; (2) substrate analogues which are unable to play the role of electron donor do not show any protective effect, despite the fact that some of them associate well with the enzyme (for instance, thionicotinamide NADH or malonate); (3) resistance of the respiratory chain to lytic enzymes, other conditions being equal, depends on the rate of electron transfer.

Of special interest are the results showing that to stabilize succinate oxidase, electron transfer across the whole multi-enzyme system, or at least across phospholipase-sensitive sites[15,16], is necessary. Incubation of particles with succinate and TMPD in the absence of oxygen (*i.e.* under the conditions when the electrons are transferred across flavoprotein F_s, nonheme iron protein, coenzyme Q and cytochrome *b*) does not increase their resistance to cobra venom. Similarly, electron transfer across cytochromes c_1 and *c* and cytochrome oxidase is not accompanied by stabilization of the succinate oxidase system. In Fig. 5 the data are summarized pertaining to the question of the relationship between electron transfer and the resistance of succinate oxidase system to cobra venom phospholipase.

The mechanism of the protective effect is not clear yet. There is no doubt that greater resistance to lytic enzymes testifies to some structural changes in the respiratory chain when electron transfer takes place. According to numerous data the components of the respiratory chain have a different structure in oxidized and reduced states[17–22]. The results of this work suggest that there is some peculiar conformation of the respiratory chain which it acquires at the moment of electron transfer. This conformation differs from the conformation of the oxidized and reduced forms of the multi-enzyme system by its resistance to lytic enzymes.

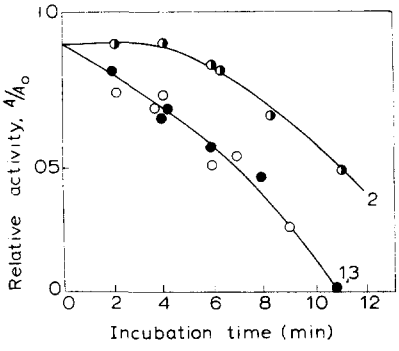

Fig. 4. Resistance of cytochrome oxidase to cobra venom under the conditions of electron transfer. The ultrasonic submitochondrial particles were incubated at 20 °C as described above. 1, incubation without succinate and TMPD; 2, in the presence of 20 mM succinate, aerobic conditions; 3, 0.1 mM TMPD and 1 mM ascorbate, aerobic conditions.

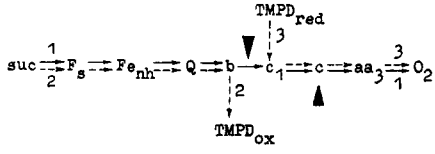

Fig. 5. Relationship between electron transfer and resistance of succinate oxidase system to cobra venom phospholipase. Electron transfer *via* Pathway 1 (solid arrows) is accompanied by an increase in resistance of succinate oxidase system to the action of cobra venom phospholipase. Electron transfer *via* Pathways 2 and 3 (broken arrows) do not enhance the stability of succinate oxidase. Thick arrows show the sites at which phospholipase attacks succinate oxidase system.

The nature of conformational changes associated with electron transfer can be judged by taking into account the sites where lytic enzymes attack the respiratory chain. This problem is mostly studied at the present time for the case of inactivation of the respiratory chain by cobra venom phospholipase. Minakami *et al.*[15] and later Luzikov *et al.*[16] showed that the succinate oxidase has two sites sensitive to the action of phospholipase A. The first one (the most labile) is localized between cytochromes c_1 and aa_3 (at the cytochrome *c* level) and the second one between cytochromes *b* and c_1 (see Fig. 5). Stabilization of the respiratory chain signifies that during electron transfer the indicated sites prove inaccessible to phospholipase molecules. It may be assumed that at a certain moment of the transfer of each electron *via* the respiratory chain when some carriers are already reduced while others are still oxidized the character of their interaction changes. Then a rearrangement of the system as a whole takes place, resulting particularly in screening the

phospholipase-sensitive sites from the surrounding medium. In this case the higher the rate of electron transfer the more probable the appearance of a stable conformation of the respiratory chain. These concepts are in accord with the scheme of electron transfer *via* Complex III previously suggested by Baum *et al.*[23].

It should be emphasized that the ability to be stabilized due to functioning is a distinguishing feature of the whole respiratory chain which its fragments do not possess. It was established earlier that the rate of inactivation of NADH: cytochrome *c* oxidoreductase by cobra venom phospholipase does not decrease in the presence of NADH and cytochrome *c* (ref. 1). This fragment is not affected by cobra venom when it is a part of the functioning respiratory chain. As was demonstrated in the present work the above is also true of the cytochrome oxidase fragment. Now there are grounds to believe that it is only quite a definite combination of the components forming the unique structure of the respiratory chain that makes it withstand the attack of phospholipase A and possibly other agents[1,2,24–27].

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