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COMPARATIVE STUDY OF THERMAL DEGRADATION OF ELECTRON TRANSFER PARTICLES AND RECONSTITUTED RESPIRATORY CHAIN

RELATION OF ELECTRON TRANSFER TO REACTIVATION OF SUBMITOCHONDRIAL PARTICLES

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SUMMARY

The thermal degradation of alkaline electron transfer particles and a respiratory chain reconstituted from simple enzyme complexes has been studied. It is shown that the reconstituted system loses its NADH oxidase activity rapidly and irreversibly at 38°. Electron transfer particles are also readily inactivated, but their NADH oxidase activity is restored completely after the addition of NADH under aerobic conditions. The NADH:cytochrome *c* oxidoreductase activity of submitochondrial particles undergoes similar changes, whereas the NADH: ferricyanide oxidoreductase and cytochrome oxidase activities do not change at all. Heating electron transfer particles results in a sharp drop in the steady-state levels of reduced cytochromes *b*, $c(+c_1)$, and aa_3 at 6°, with NADH as the substrate. These results mean that one of the thermolabile electron transfer particles sites precedes cytochrome *b* in the respiratory chain.

Preheated electron transfer particles are more sensitive to the action of chymotrypsin and potassium oleate than are the intact particles under the same conditions. Oleate prevents reactivation and causes substantial changes in the steady-state levels of reduced cytochromes in the preheated particles. Reactivation is accompanied by an increase in resistance of the particles to oleate and chymotrypsin. These observations suggest that the structure of electron transfer particles becomes slightly looser on heating, and is restored on reactivation.

To reactivate the NADH oxidase system of submitochondrial particles NADH and oxygen are needed. Zn^{2+} inhibits electron transfer and thus prevents reactivation. Incubation of preheated particles with an excess of NADH and exogenous oxidized cytochrome *c* under anaerobic conditions does not result in complete recovery of NADH oxidase activity. Taking into account these results it must be concluded that reactivation is related to electron transfer through the entire NADH oxidase system, including cytochrome oxidase.

INTRODUCTION

MORRIS AND KING[1] have established that heart muscle preparations lose their NADH oxidase activity comparatively slowly when heated. However, as follows from

a number of our previous papers, a respiratory chain reconstituted from simple enzyme complexes is more labile[2,3]. In this paper it will be shown that a non-fragmented respiratory chain is only seemingly stable. Experimental results will be given according to which alkaline electron transfer particles lose their NADH oxidase activity rapidly on heating, but are easily reactivated by NADH under aerobic conditions. The NADH oxidase activity of preheated electron transfer particles increases gradually during electron transfer through the respiratory chain. Inactivation and reactivation of the particles are most probably caused by reversible changes of their conformation.

According to the data of a number of authors, the properties of a reconstituted respiratory chain resemble to some extent the properties of an intact multi-enzyme system[4–7]. The results of the present study suggest that these systems are not identical.

MATERIALS AND METHODS

In this work we used alkaline electron transfer particles isolated from beef heart mitochondria according to the method of CRANE *et al.*[8]. Some experiments, specified in the paper, were carried out with ultrasonic submitochondrial particles[9]. The NADH oxidase activity of the electron transfer particles, measured spectrophotometrically, was 3.0–5.0 μmoles of NADH oxidized per min per mg particle protein at 38°. When we assayed the activity of, or incubated, electron transfer particles, the medium contained 80 mM of potassium phosphate (pH 7.4) and EDTA (1 mM). The reaction was started by adding particles (0.01–0.10 mg protein per ml). The extinction coefficient for NADH at 340 nm ($\Delta\varepsilon_{340\,nm}$) was taken to equal 6.2 $mM^{-1} \cdot cm^{-1}$. The NADH: cytochrome *c* oxidoreductase, NADH: ferricyanide oxidoreductase, and cytochrome oxidase activities of electron transfer particles were measured as described earlier[1].

The respiratory chain was reconstituted from NADH: cytochrome *c* oxidoreductase, succinate: coenzyme Q oxidoreductase, cytochrome oxidase and cytochrome *c*. The NADH: cytochrome *c* oxidoreductase (complexes I + III) was isolated by the method of HATEFI *et al.*[10], succinate: coenzyme Q oxidoreductase (complex II) by the method of ZIEGLER AND DOEG[11], and cytochrome oxidase (complex IV) by the method of GRIFFITHS AND WHARTON[12].

Concentrated solutions of the enzymes (15–25 mg protein per ml) and cytochrome *c* were mixed at 0°, and the mixtures were diluted with 0.05 M Tris–HCl (pH 8.0) containing 0.005 M histidine and 0.66 M sucrose (Tris–histidine–sucrose) to a concentration of 0.04 mg cytochrome oxidase protein per ml. The ratio of the complexes I + III, II, and IV during reconstitution was 2.5:1.0:0.6 with respect to protein content. As HATEFI *et al.*[4] have shown, at this ratio the specific activity of the reconstituted system is maximal. Cytochrome *c* was added in an amount of 1.0 mg/mg cytochrome oxidase protein. The NADH oxidase activity of the reconstituted system, measured as described earlier[4], amounted to 19–23 μmoles NADH oxidized per min per mg cytochrome oxidase protein at 38°. The succinate oxidase activity was 14 μmoles succinate oxidized under the conditions indicated above. The reconstituted respiratory chain was incubated in Tris–histidine–sucrose (see above) or in 20 mM potassium phosphate (pH 7.4–7.8) containing EDTA (1.0 mM).

Enzyme activities were measured with an EPS-3 recording spectrophotometer (Hitachi) and a PO-4 polarograph (Radiometer) with a platinum electrode. Cyto-

chrome reduction was followed with a dual-wavelength spectrophotometer (DSF-1M, USSR, or Hitachi, Model 356) at the wavelengths indicated in the figure legends.

Reagents

NADH was obtained from Reanal or Calbiochem, cytochrome *c* (Type III) from Sigma or British Drug Houses, antimycin A from Calbiochem. Crystalline chymotrypsin was obtained from the Leningrad factory of medical preparations. Oleic acid was purified before use by vacuum distillation.

RESULTS

Thermal degradation of electron transfer particles and reconstituted respiratory chain

Fig. 1 gives results concerning thermal inactivation of electron transfer particles and the reconstituted respiratory chain. Evidently the particles are stable to heating for several hours. Under the same conditions the NADH oxidase activity of the reconstituted respiratory chain disappears practically completely in 30 min.

Further experiments showed, however, that electron transfer particles also undergo substantial changes when heated. These changes show up distinctly when the NADH oxidase activity of electron transfer particles incubated at 38° is assayed at 10–15°. In contrast to the intact particles, the degraded ones at first show only a slight NADH oxidase activity which increases in time and reaches a certain limiting value. As shown in Fig. 2, only 5 min incubation of electron transfer particles at 38° decreases their activity substantially; however, the latter is quickly recovered when NADH is added. The preheated particles are reactivated practically completely even after 90 min incubation at 38°. The results of the experiment are independent of the method used for measuring NADH oxidase activity. A lag period is observed, whether

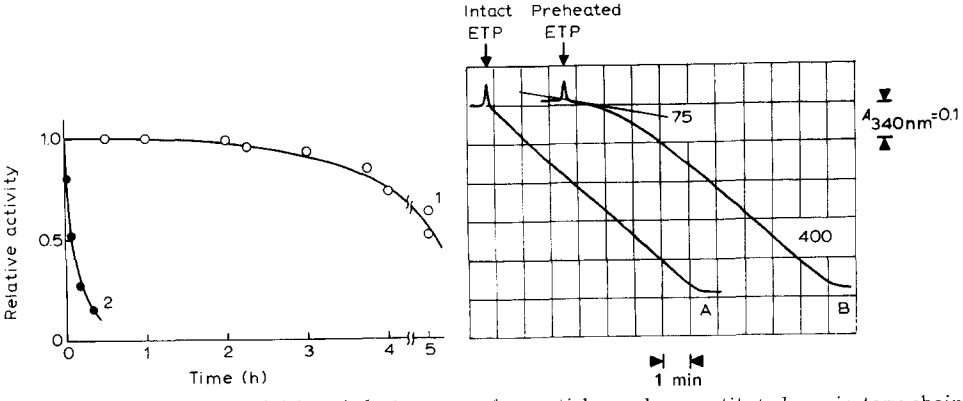

Fig. 1. NADH oxidase activities of electron transfer particles and reconstituted respiratory chain heated at 38° for various times. The reaction mixture consisted of 80 mM potassium phosphate (pH 7.8) and 1 mM EDTA. The concentration of total protein during heating was 0.03–0.09 mg/ml. All measurements were made at 38°. A/A_0 is the ratio of the current to the initial specific activity. Curve 1, electron transfer particles. Curve 2, reconstituted respiratory chain (complexes I + II + III + IV).

Fig. 2. Oxidation of NADH in the presence of intact and preheated electron transfer particles (ETP). The reaction mixture consisted of 80 mM potassium phosphate (pH 7.4), 1 mM EDTA, 0.13 mM NADH, and 0.025 mg particle protein per ml. Temperature 10°. Reaction was started by the addition of particles. Activities in this figure are expressed as a nmoles of NADH oxidized per min per mg protein. Trace A, intact electron transfer particles. Trace B, electron transfer particles preincubated at 38° for 5 min.

the NADH oxidation is recorded spectrophotometrically (Fig. 2) or the oxygen consumption is recorded by a polarographic procedure.

The reactivation rate of preheated electron transfer particles depends substantially on the temperature. At 25° reactivation is complete in less than 2 min, whereas at 38° it proceeds so fast that only the maximal NADH oxidase activity can be recorded. For this reason information on the behaviour of electron transfer particles at an elevated temperature cannot be considered sufficiently complete if NADH oxidase activity was also assayed at a high temperature (see Fig. 1).

Unlike electron transfer particles, reconstituted respiratory chains are not reactivated substantially after heating at 38°. The results shown in Fig. 1 correspond to the maximal values of the NADH oxidase activities of electron transfer particles and the reconstituted system after being heated for different times.

A rise in temperature during incubation increases the fraction of irreversibly degraded electron transfer particles which can no longer be reactivated in the presence of NADH. Irreversible degradation becomes substantial above 42 or 43°. Thus, after incubation of the particles for 25 min at 47°, only 12 % of the initial NADH oxidase activity is recovered.

No reactivation effect is observed during the assay of the succinate oxidase activity of electron transfer particles preheated for 30 min at 40° in 80 mM potassium phosphate (pH 7.4). In these conditions the rate of oxygen consumption increases 3–4-fold at 12° compared with intact electron transfer particles, and no lag period is observed. It was shown earlier that incubation of Keilin–Hartree particles in potassium phosphate increases their succinate oxidase activity[13]. This effect is evidently attributable to activation of the succinate dehydrogenase in the respiratory chain[14].

Change in NADH:ferricyanide oxidoreductase, NADH:cytochrome c oxidoreductase, cytochrome oxidase, and NADH oxidase activities upon heating and reactivation of electron transfer particles

Some idea of the mechanism of degradation can be obtained by comparing the NADH oxidase, NADH:cytochrome *c* oxidoreductase, cytochrome oxidase, and NADH:ferricyanide oxidoreductase activities of preheated submitochondrial particles. The curves in Fig. 3 show how these activities change during measurement. The points on the curves correspond to the rates of oxidation or reduction of NADH, cytochrome *c*, and potassium ferricyanide at the moments indicated. It is evident from Fig. 3 that heating the particles for 20 min at 43° reduces their NADH oxidase activity by 90 %. The activity increases if there is NADH in the medium, and after 4.0 or 4.5 min reaches the activity of the intact particles. The NADH:cytochrome *c* oxidoreductase activity of the same sample changes less. At the initial moment of measurement it is not less than 50 % of the activity of the intact electron transfer particles and is recovered completely in 1.5–2.0 min. The results given are characteristic of alkaline particles only. With ultrasonic particles measurements of NADH:cytochrome *c* oxidoreductase and NADH oxidase activities result in practically identical reactivation curves*. Finally, the NADH: ferricyanide oxidoreductase and cyto-

* The NADH: cytochrome *c* oxidoreductase activity of alkaline electron transfer particles was inhibited not more than 65 % by antimycin A (2.0 μg/mg of protein), whereas the analogous activity of ultrasonic particles was depressed by 85–90 %. NADH oxidase activity was displayed neither by alkaline nor by ultrasonic particles in the presence of antimycin A.

chrome oxidase activities of both kinds of particle do not decrease on heating. Measurements of these activities for the preheated particles show no lag period in the reactions of ferricyanide reduction and reduced cytochrome *c* oxidation.

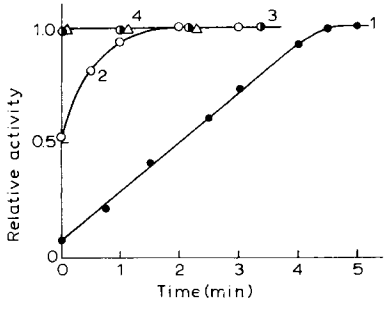

Fig. 3. Changes in NADH oxidase, NADH: cytochrome *c* oxidoreductase, NADH: ferricyanide oxidoreductase and cytochrome oxidase activities of preheated electron transfer particles. Electron transfer particles were heated for 20 min at 43° in 80 mM potassium phosphate (pH 7.4) containing 1 mM EDTA. Measurements were made at 10° as described in Fig. 2. Corresponding activities of intact electron transfer particles were taken as A_0. Curve 1, NADH oxidase activity (A_0 was 200 nmoles of NADH oxidized per min per mg protein); 2, NADH: cytochrome *c* oxidoreductase activity (A_0 was 20 nmoles of cytochrome *c* reduced per min per mg protein) in the presence of 2 mM KCN; 3, NADH: ferricyanide oxidoreductase activity (A_0 was 800 nmoles of potassium ferricyanide reduced per min per mg protein); 4, cytochrome oxidase activity (A_0 was 80 nmoles of cytochrome *c* oxidized per min per mg protein).

The observations described in this section are in accord with those published previously. In particular, it has been reported that NADH dehydrogenase and cytochrome oxidase linked to particles are comparatively stable[1,15]. It has also been established that isolated cytochrome oxidase (complex IV) is inactivated slowly by heating in dilute solutions, whereas NADH: cytochrome *c* oxidoreductase (complexes I + III) is labile[2]. According to MORRIS AND KING[1] the NADH oxidase and NADH: cytochrome *c* oxidoreductase activities of a heart muscle preparation decrease on heating at practically the same rate. In this respect NADH oxidase reconstituted

TABLE I

STEADY-STATE LEVELS OF REDUCED CYTOCHROMES DURING RESPIRATION OF ELECTRON TRANSFER PARTICLES IN THE PRESENCE OF NADH AND SUCCINATE

Steady-state levels of reduced cytochromes *b*, $c(+c_1)$ and aa_3 (percent completely reduced by substrate) were determined with a dual-wavelength spectrophotometer at the wavelengths indicated in the table. Conditions were as for Fig. 4. Particles were preheated for 30 min at 40° before the measurements. ETP: electron transfer particles.

	Substrate							
	NADH				*Succinate*			
	Intact ETP		*Preheated ETP*		*Intact ETP*		*Preheated ETP*	
	6°	17°	6°	17°	6°	25°	6°	25°
Cytochrome *b* (562–575 nm)	36	45	12	44	—	37	—	—
Cytochrome $c(+c_1)$ (550–540 nm)	31	36	19	31	41	28	38	—
Cytochrome aa_3 (605–630 nm)	21	24	6	23	35	25	34	27

from simple enzyme complexes behaves somewhat differently: it is inactivated perceptibly more rapidly than isolated NADH:cytochrome *c* oxidoreductase[2].

These findings and the results of the present study suggest that electron transfer particles have at least one thermally labile site located within the NADH:cyto-

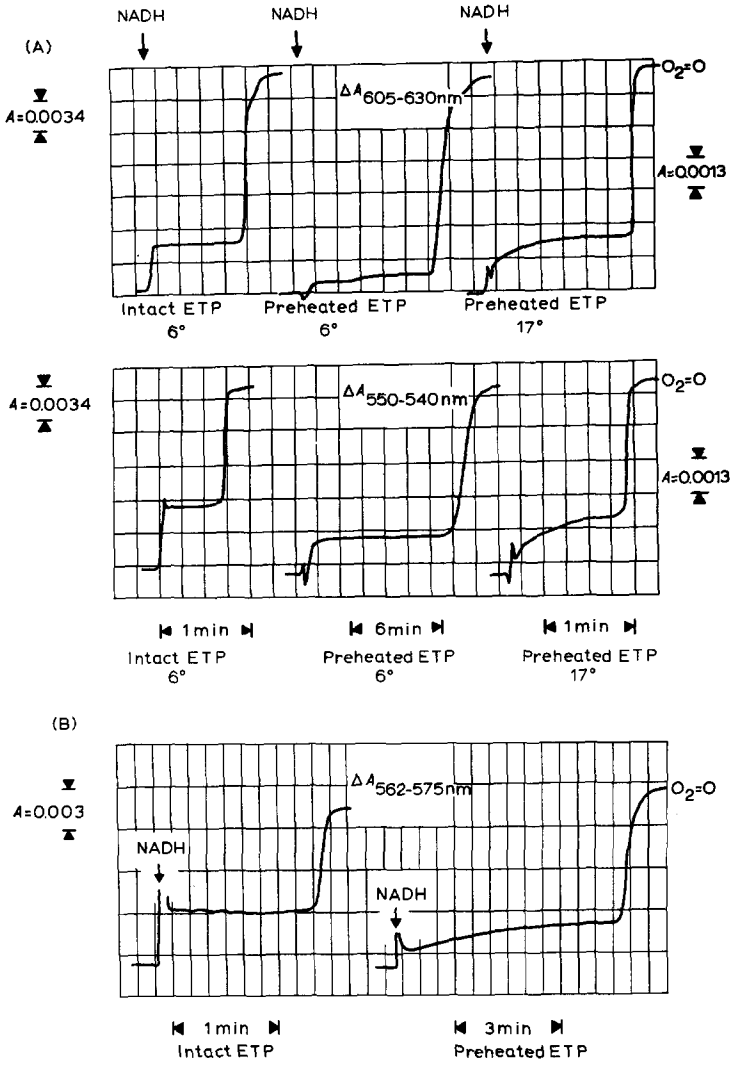

Fig. 4. Steady-state analysis of cytochromes in intact and preheated electron transfer particles (ETP). A. The reaction mixture consisted of 80 mM potassium phosphate (pH 7.4), 1 mM EDTA and 1.7 mM NADH. 3.0 mg particle protein per ml was injected into the cuvette for measurements at 6° (1.15 mg protein per ml for measurements at 17°). Reaction was started by adding NADH. Reduction (upward deflection of traces) of cytochromes $c(+c_1)$ and aa_3 was followed at 550–540 and 605–630 nm, respectively[38]. Measurements were made with a DSF-1M dual-wavelength spectrophotometer (optical path 0.86 cm). When necessary, electron transfer particles were preincubated for 30 min at 40°. B. Conditions were as for Fig. 4A, except that electron transfer particles concentration was 2.0 mg protein per ml. Measurements were made with a Hitachi 356 dual-wavelength spectrophotometer at 7°. For details see text.

chrome *c* oxidoreductase system, or more precisely, between NADH dehydrogenase and cytochrome *c*.

Steady-state analysis of intact and preheated electron transfer particles

Some essential changes occurring in electron transfer particles upon heating can be detected by a comparative steady-state analysis of intact and preheated particles. Some of the results obtained at 6° are listed in Table I. During respiration in the presence of NADH the steady-state levels of reduced cytochromes *b*, $c(+c_1)$, and aa_3 in the intact electron transfer particles are 35–37, 30–31 and 20–23 %, respectively. These values resemble those for phosphorylating submitochondrial particles treated with an uncoupler[16]. After the electron transfer particles have been heated, the steady-state levels of all the reduced cytochromes decrease, and it is evident from Fig. 4A that this decrease is very substantial for cytochrome aa_3 and less noticeable for cytochrome $c(+c_1)$. The experimental data characterize the steady-state of the preheated particles fairly accurately, since at 6° reactivation occurs slowly. It follows specifically from these data that a heat-sensitive site precedes cytochrome *b* in the respiratory chain.

The steady-state levels of reduced cytochromes change little in intact electron transfer particles when the temperature is raised from 6 to 17°. A different picture is observed for preheated particles. Fig. 4A shows that in this case the steady-state level of reduced cytochrome aa_3 at 17° is lower at the initial moment than for intact electron transfer particles, but increases gradually to the value characteristic of the latter (see Table I). Similar results are obtained when the reduction of cytochromes *b* and $c(+c_1)$ are recorded. The change in the steady-state of preheated particles in the presence of NADH at 17° is evidently due to their reactivation. It should, however, be noted that the increase in NADH oxidase activity and recovery of the initial steady-state characteristics occur at different rates. At 17° the former is complete in an average of 2.0–2.5 min, while the latter takes not more than 0.8–1.0 min. In some electron transfer particles preparations preincubated at 40° the steady-state levels of the reduced cytochromes increase gradually almost to the normal values at as low a temperature as 7° (Fig. 4B).

Preheating of electron transfer particles for 30 min at 40° causes no noticeable change in the steady-state levels of the reduced cytochromes $c(+c_1)$ and aa_3 at 6° when succinate is used as the substrate. Yet incubation of electron transfer particles in a phosphate buffer (pH 7.4) at 40° is accompanied by an almost 3-fold increase in succinate oxidase activity.

Effect of potassium oleate on reactivation of electron transfer particles

This section gives results which show that oleate retards or completely depresses reactivation of electron transfer particles. At a concentration of 20 μM oleate inhibits the NADH oxidase system of intact particles by 34 %. The same amount of oleate considerably retards the reactivation of particles preheated for 30 min at 40°. In this event the activity equal to that obtained for intact particles treated with 20 μM oleate is not reached in 3 min (Fig. 5). In the absence of oleate, other conditions being equal, reactivation of electron transfer particles is complete in 20 sec. When the oleate concentration is 30 μM, preheated particles display no noticeable ability to oxidize NADH at all in 3 min, though under the same conditions the intact particles still retain 31 % of their initial NADH oxidase activity.

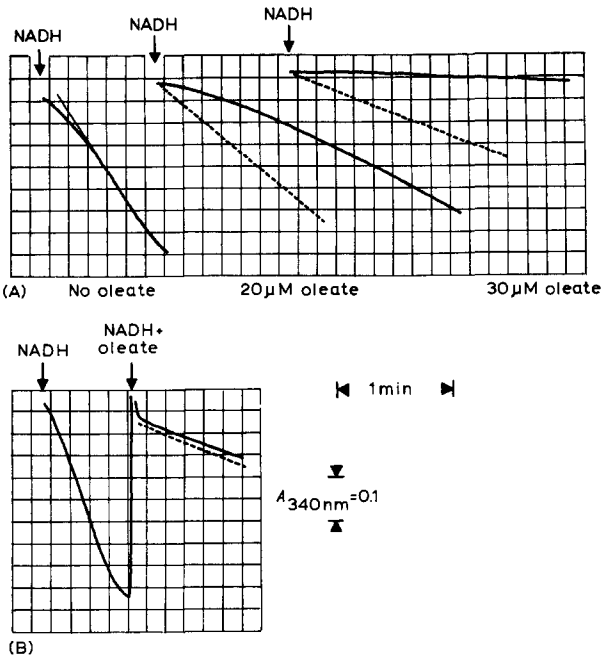

Fig. 5. Effect of potassium oleate on reactivation of preheated electron transfer particles. A. Reaction mixture of 2.5 ml containing 0.082 mg particle protein, 80 mM potassium phosphate (pH 7.4), 1 mM EDTA, 0.13 mM NADH and various amounts of oleate. Reaction was started by adding NADH. Temperature 25°. Broken traces show oxidation of NADH in the presence of electron transfer particles that had not been heated. Solid traces, the same in the presence of electron transfer particles preheated for 30 min at 40°. B. Reaction mixtures were as described for A, except that the amount of particles was 0.085 mg protein per ml. Potassium oleate (40 μM) was injected into the cuvette after NADH. Temperature 25°.

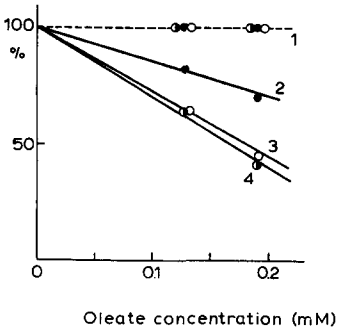

Fig. 6. Steady-state analysis of cytochromes in electron transfer particles (intact and preheated) in the presence of potassium oleate. The reaction mixture consisted of 80 mM potassium phosphate (pH 7.5), 1 mM EDTA, 1.6 mM NADH, 0.6 mg protein per ml and various amounts of oleate. The reaction was started by adding NADH. Temperature 25°. Reduction of cytochromes *b*, $c(+c_1)$, and aa_3 was measured with a dual-wavelength Hitachi spectrophotometer (model 356) at 562–575, 550–540, and 605–630 nm, respectively. Steady-state levels of reduced cytochromes were related to those measured in the absence of oleate, taken as 100%. Curve 1, cytochromes *b*, $c(+c_1)$, aa_3 for intact particles; Curve 2, cytochrome $c(+c_1)$; Curve 3, cytochrome *b*; Curve 4, cytochrome aa_3.

It follows from Fig. 6 that the addition of oleate to intact electron transfer particles involves no change in the steady-state levels of reduced cytochromes *b*, $c(+c_1)$ and aa_3, though the NADH oxidase activity decreases 2-fold at the oleate concentration indicated. Similar results were obtained previously by TYLER AND ESTABROOK[17], who found that certain organic solvents (glycerol, ethylene glycol, *etc.*) inhibit the respiratory chain "unspecifically". These solvents lower the rate of oxidation of NADH, but do not change the steady-state of the particles. Evidently, in intact electron transfer particles the action of solvents, like that of oleate, is not localized at any strictly definite parts of the respiratory chain.

On the contrary, treating preheated particles with oleate substantially decreases the content of the reduced forms of cytochromes in the steady-state when NADH is used as the substrate (Fig. 6). Here, the steady-state levels of the reduced cytochromes *b* and aa_3 decrease more rapidly. Since the measurements were made at 25°, when reactivation occurs very quickly (Fig. 5), the steady-state characteristics of the intact particles practically coincide with those of the preheated particles in the absence of oleate.

These results show that preheating of electron transfer particles involves not only a sharp increase in their sensitivity to the action of oleate, but also a change in the mechanism of this action. It is evident from Figs. 4 and 6 that oleate (0.33 μmole/mg protein) fixes the changes caused in the particles by heating. It was indicated above that the steady-state levels of reduced cytochromes in electron transfer particles preincubated at 40° decrease sharply. This can be observed only at a low temperature (5–7°), since heating the solution to 17° in the presence of NADH results in rapid recovery of the steady-state characteristics (Fig. 4). On the contrary, after treatment of preheated particles with oleate, the steady-state levels of reduced cytochromes do not return to normal even at 25° (Fig. 6). The high effectiveness of oleate on preheated particles is evidently related to loosening of their structure upon heating, which facilitates access of fatty acid molecules to definite sites of the respiratory chain.

It is noteworthy that oleate displays a substantial effect only when added to the preheated particles before NADH (Fig. 5). After reactivation, electron transfer particles are inactivated by oleate to the same degree as intact particles. This shows that the structure of electron transfer particles is restored as a result of treatment with NADH under aerobic conditions.

Inactivation of intact, preheated, and reactivated electron transfer particles by chymotrypsin

Results similar to those described above were obtained in a study of the action of chymotrypsin on intact, preheated, and reactivated electron transfer particles. As is evident from Fig. 7, preheating particles at 38 or 47° lowers their resistance to chymotrypsin. This difference between intact and preheated particles disappears if both kinds of particle are incubated for several minutes in the presence of NADH before chymotrypsin is added. The change in sensitivity to the action of chymotrypsin after heating and reactivation is evidence of reversible changes in the structure of certain components of the respiratory chain.

Role of NADH and oxygen in reactivation of electron transfer particles

Attention is drawn to the fact that reactivation of preheated particles does not occur spontaneously when the temperature is lowered. A necessary condition for the

recovery of NADH oxidase activity is the presence of NADH and oxygen in the medium. Fig. 8 (Trace A) presents the results of measurement of the NADH oxidase activity of electron transfer particles that were first heated and then incubated for a considerable time with NADH under anaerobic conditions. In this experiment, to

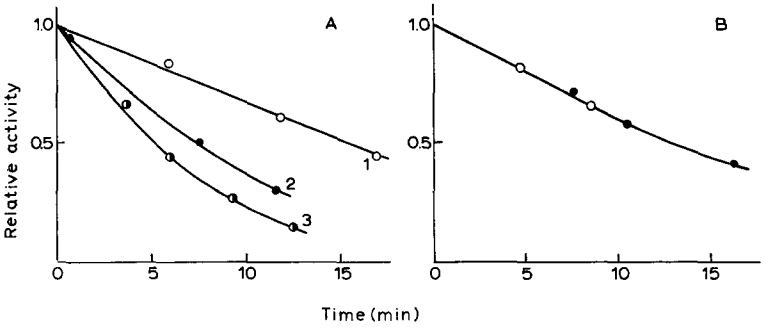

Fig. 7. Inactivation of intact, preheated, and reactivated electron transfer particles with chymotrypsin. A. Particles (0.10 mg protein per ml) were incubated with chymotrypsin (30 mg/ml) in 80 mM potassium phosphate (pH 7.4) containing 1 mM EDTA at 25°. Curve 1, intact electron transfer particles; 2, particles preheated for 30 min at 38°; 3, particles preheated for 5 min at 47°. B. Conditions were as for A, except that 0.033 mg particle protein per ml and 13.2 mg of chymotrypsin per ml were present. Temperature 30°. Chymotrypsin was added to the solution 3 min after the addition of 0.13 mM NADH. ●, intact electron transfer particles; ○, particles preheated for 30 min at 40°.

remove traces of oxygen, the NADH and intact particles were introduced into a nitrogen-saturated phosphate buffer, and then an equal amount of preheated particles was added. The activity of a sample taken from the solution after 1 h incubation was measured under standard conditions at 10°. At first, NADH oxidation was caused only by intact electron transfer particles. The rate of oxidation gradually increased 2-fold, the maximal NADH oxidase activity referred to total protein becoming equal to the specific activity of the intact electron transfer particles. Unlike the previous example, even a short (5 min) incubation with NADH in the presence of oxygen, other conditions being equal, results in complete restoration of NADH oxidase activity of the preheated particles (Fig. 8, Trace B). Experiments with preheated ultrasonic particles showed that their NADH:cytochrome *c* oxidoreductase activity is not restored when the solution contains NADH, oxygen and CN^-. These observations suggest that electron transfer is a necessary condition for restoration of the NADH:cytochrome *c* oxidoreductase, and hence, the NADH oxidase system.

Electron transfer to oxidized cytochrome *c* is insufficient for complete reactivation of electron transfer particles. Fig. 8 gives the results of an experiment in which preheated particles were incubated at 26° with NADH and exogenous cytochrome *c* in a solution saturated with argon. Both alkaline and ultrasonic submitochondrial particles were used. Antimycin A depressed the NADH:cytochrome *c* oxidoreductase activity of the latter by 90% (see above), and it may therefore be supposed that here electron transfer to the exogenous cytochrome *c* went predominantly *via* cytochromes *b* and c_1. As mentioned above, reactivation of electron transfer particles is complete at 26° in the presence of NADH and oxygen in not more than 1 min. However, even 7 min incubation of the particles with NADH and cytochrome *c* in the

absence of oxygen did not completely reactivate them, though almost all the cytochrome *c* was reduced. On being transferred to the cuvette of a spectrophotometer after such incubation, electron transfer particles showed a comparatively small NADH oxidase activity at first which gradually increased 1.7–2.0-fold (Fig. 8, Trace D). A similar picture was observed when the preheated particles were incubated with NADH in a medium saturated with argon and not containing exogenous cytochrome *c* (Fig. 8, Trace C). Unlike NADH oxidase activity, NADH:cytochrome *c* oxidoreductase activity is completely restored by treatment of the preheated particles with cytochrome *c* and NADH under aerobic conditions. When the activity of reactivated particles was recorded no lag period could be observed in the oxidation of NADH by cytochrome *c*. The above results suggest that complete reactivation of preheated particles requires electron transfer throughout the entire respiratory chain, including cytochrome oxidase.

Two assumptions that may account for this interesting phenomenon are worthy of discussion. First, it may be thought that a fairly high rate of electron transfer is

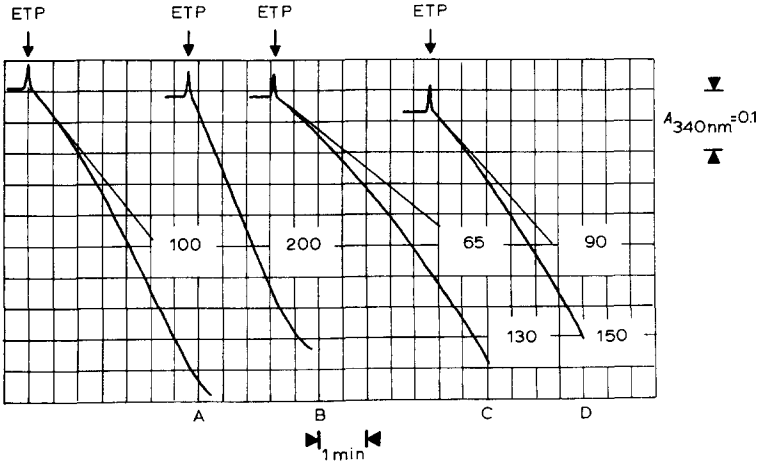

Fig. 8. Effect of oxygen on reactivation of preheated electron transfer particles (ETP). A. electron transfer particles (6.0 mg protein per ml) were heated for 30 min at 40° in deaerated 80 mM potassium phosphate (pH 7.4) with EDTA (1.0 mM). The suspension was mixed at 0° with a deaerated solution containing, apart from potassium phosphate, EDTA, 2.8 mM NADH and intact electron transfer particles. After 5 min the resulting mixture of intact electron transfer particles (1.0 mg protein per ml) and preheated particles (1.0 mg protein per ml) was heated to 20° and incubated for 60 min. 0.1 ml of this solution was taken for an estimate of NADH oxidase activity. Measurements were made at 10°. Activities are expressed as nmoles of NADH oxidized per min per mg protein. B. A mixture of intact and preheated electron transfer particles was incubated for 5 min in the presence of NADH under aerobic conditions before the NADH oxidase activity was measured (see Fig. 8A). C. 2.0 ml of 80 mM potassium phosphate (pH 7.5) with 1.0 mM EDTA was deaerated in a test-tube by passing argon through it for 20 min. To this solution were added 0.2 ml of an electron transfer particles suspension (33 mg protein ml) preincubated for 30 min at 40°, and 0.2 ml of a deaerated solution of NADH (58 mM). The mixture was held at 20° for 10 min, after which a sample (0.2 ml) was taken and its NADH oxidase activity was measured under standard conditions at 8°. D. After 10 min incubation of electron transfer particles with NADH (see above) 30 mg of oxidized cytochrome *c* were added, and the suspension was incubated for another 7 min at 26°. NADH oxidase activity was measured in a 0.2-ml sample at 8°. This electron transfer particles preparation catalysed the oxidation of 100 nmoles of NADH per min per mg protein at 26° in the presence of KCN and cytochrome *c* (0.7 mg/ml).

required for complete recovery of NADH oxidase activity of the system, and that this rate is not attained when exogenous cytochrome *c* is the electron acceptor*. Under the conditions indicated the degraded multi-enzyme system is only partly restored, but it nevertheless becomes capable of reducing the exogenous cytochrome *c* at a maximal rate. On the other hand, it is not impossible that in an active NADH oxidase system cytochrome oxidase must be oriented in a definite manner relative to the other components of the system. In particular, it has been shown previously[37] that reconstitution of NADH oxidase (complexes I + III + IV) involves a sharp decrease in NADH:ferricyanide oxidoreductase activity of complex I. This effect is probably due to screening of the active centre of NADH dehydrogenase by cytochrome oxidase (complex IV). If this is so, electron transfer through cytochrome oxidase may be necessary for full recovery of the structure of the multi-enzyme system. The mechanism of restoration of preheated particles requires further study before anything final can be said about its details.

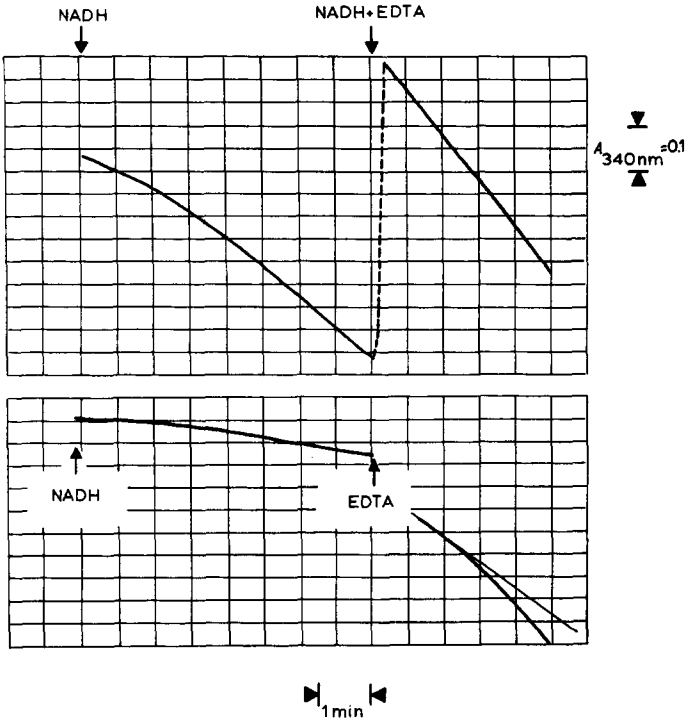

Fig. 9. Effect of Zn^{2+} on reactivation of preheated electron transfer particles. Oxidation of NADH (0.13 mM) in 80 mM potassium phosphate (pH 6.5) was recorded at 12°. Electron transfer particles (2.0 mg protein per ml) were preheated for 30 min at 40° in 80 mM potassium phosphate (pH 7.5). Reaction was started by the addition of NADH. Upper trace shows oxidation of NADH in the absence of Zn^{2+}. For the lower trace the medium contained Zn^{2+} (0.8 mM). EDTA (6.6 mM) or EDTA and NADH (0.13 mM) were injected into the cuvette at the times indicated.

* With alkaline electron transfer particles the rate of oxidation of exogenous cytochrome *c* is about 10% of the rate of NADH oxidation by oxygen (see Fig. 3). For ultrasonic particles this rate is up to 30%.

Effect of Zn^{2+} on reactivation of electron transfer particles

Zn^{2+} inhibits respiration[18,19] by interrupting electron transfer at several points of the respiratory chain. Fig. 9 shows that Zn^{2+} hinders reactivation of preheated electron transfer particles in the presence of NADH and oxygen. In this series of experiments 80 mM potassium phosphate (pH 6.5) was used as the medium. Slight acidification of the solution compared with the previous experiments was necessary to increase the effectiveness of Zn^{2+} action on electron transfer particles. This was because at pH 7.5 zinc acetate inhibits particles by not more than 75 %, as special investigations have shown. However, at pH 6.5 Zn^{2+} is capable of depressing the NADH oxidase activity of electron transfer particles to an extent of 90–94 %, whereas decreasing the pH to 6.5 in itself does not lower the activity of the intact particles perceptibly.

It follows from Fig. 9 that preheated particles are completely reactivated in the presence of NADH in 3.5–4.0 min (upper curve). If the solution contains Zn^{2+} besides NADH, the rate of oxidation of the substrate during the same period of time remains very low (lower curve). After the addition of EDTA the rate increases, gradually approaching the value characteristic of intact particles. This experiment shows that EDTA practically instantaneously and completely stops the inhibiting action of Zn^{2+} on intact particles. Hence, the slow increase in NADH oxidase activity of preheated particles is most probably due to reactivation of the latter, which begins only after Zn^{2+} has been removed. The more completely the NADH oxidase activity of particles is depressed, the more distinct is the effect of Zn^{2+} on their reactivation.

DISCUSSION

It follows from this work that, when heated, electron transfer particles rapidly lose their ability to catalyse NADH oxidation with oxygen. However, they are easily reactivated in the presence of NADH unless heated for too long.

According to published data, NADH acts on the respiratory chain in two ways. On the one hand, NADH is capable of specifically making the structure of NADH dehydrogenase linked to a respiratory chain labile by causing fragmentation of the enzyme and the appearance of highly reactive sulphydryl groups[15,20–23]. On the other hand, we established in earlier papers that, when catalysing the oxidation of NADH with oxygen, the respiratory chain acquires a conformation that is resistant to proteolytic enzymes and cobra venom phospholipase[3,24,25]. These effects of NADH are different in nature. In particular, the stabilizing action of NADH is displayed only during electron transfer. On the contrary, fragmentation of NADH dehydrogenase in the presence of NADH is in no way related to electron transfer[15]. Similarly, a short pretreatment of particles by NADH under aerobic conditions, which may be carried out long before the actual experiment, is sufficient to bring out the sulphydryl groups[26]. Unlike the specific action of NADH on NADH dehydrogenase, stabilization of the respiratory chain is related to a change in conformation of the multi-enzyme system as a whole. It is known, for instance, that the NADH oxidase system of electron transfer particles[25], as well as of the reconstituted respiratory chain[24], becomes stable to trypsin and phospholipase not only when NADH is oxidized, but also when succinate is used as the substrate. This experimental fact, in parallel with some other data[27–30], supports, in particular, the assumption that the conformation

of mitochondrial membranes changes at the moment of electron transfer[31–33]. Finally, predominance of either of the effects of NADH depends on its concentration. Thus, increasing the NADH concentration accelerates degradation of NADH dehydrogenase[15]. On the other hand, to stabilize the respiratory chain it is sufficient to maintain a relatively small stationary concentration of NADH in the solution.

As demonstrated above, electron transfer not only stabilizes the intact respiratory chain, but also favours the appearance of active respiratory ensembles. The results of investigations of the stability of intact, preheated, and reactivated particles against chymotrypsin and oleate are evidence that heating somewhat loosens the structure of the particles. In oxidizing NADH with oxygen the respiratory chain in the preheated particles acquires a specific conformation characterized by high enzyme activity and stability. Evidently, this process is related directly to the previously observed effect of substrates on respiratory chain formation from enzyme complexes under rigid conditions[34]. Thus, the specific influence of substrates on the respiratory chain, based on the ability of this multi-enzyme system to change its conformation upon electron transfer, may manifest itself in a variety of forms. It is not impossible that the above features of electron transfer particles behaviour may be of definite physiological sense. In particular, it may be thought that the respiratory chain is capable of existing in the active form at a temperature of about 38° only while it is functioning.

A comparative analysis of the steady-state of intact and preheated particles reveals a heat-sensitive site preceding cytochrome *b* in the respiratory chain. It follows from an estimate of the rates of NADH oxidation by various electron acceptors in the presence of preheated particles that this labile site is localized between the NADH dehydrogenase and cytochrome system. Possibly the reactivation of degraded electron transfer particles is somewhat similar to the activation of a heart muscle preparation by NADH, which was observed previously by MINAKAMI *et al.*[35]. According to these authors the "defective" site of this preparation is localized between the flavoprotein of NADH dehydrogenase and the coenzyme Q, with which our data are in agreement. There are no serious grounds at present for thinking that there are also heat-sensitive sites in the cytochrome system, which is customarily considered to be common to the NADH oxidase and succinate oxidase chains[36]. Indeed, after heating the particles we were unable to detect any decrease in the rate of succinate oxidation or any change in the steady-state of the particles when succinate was used as the substrate. Though progress has definitely been made in the study of thermal degradation of submitochondrial particles, there are certain details of the process that remain obscure. In particular, it is not clear why reactivation of the particles requires electron transfer through cytochrome oxidase if the only labile site is located before the cytochrome system. This is a problem of great interest and warrants detailed investigation.

HATEFI *et al.*[4], FOWLER AND RICHARDSON[5], KEILIN AND KING[6], and KING AND TAKEMORI[7] have shown that the properties of the reconstituted respiratory chain somewhat resemble those of the natural system. However, it follows from the present work that there are considerable differences between them. Indeed, NADH oxidase reconstituted from simple enzyme complexes is inactivated irreversibly by heating, whereas electron transfer particles can be reactivated under certain conditions. Evidently, electron transfer particles contain some stabilizing factor, the nature of which is not yet known.

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