Biochimica et Biophysica Acta, 376 (1975) 398–414

BBA 46879

FURTHER CHARACTERIZATION OF GRADIENT-FRACTIONATED SUB-MITOCHONDRIAL MEMBRANE FRAGMENTS FROM BEEF HEART MITOCHONDRIA

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(Received July 29th, 1974)

SUMMARY

We have recently reported that with a linear sucrose density gradient centrifugation two distinct types of membrane fragments, designated as X- and Y-fragments are obtained (Huang, C. H., Keyhani, E. and Lee, C. P. (1973) Biochim. Biophys. Acta 305, 455–473). Further characterization of these two membrane fragments is reported. (1) Potassium chloride at the concentration of 0.15 M extracts 7 % and 30 % of cytochrome *c* from the X- and Y-fragments, respectively. (2) When cytochrome *c* was added to the mitochondrial suspension prior to sonication, the cytochrome *c* content was increased by 6–8-fold in both X- and Y-fragments. Subsequently KCl extraction resulted in loss of cytochrome *c* by 1/4 in the X- and by 2/3 in the Y-fragments. (3) With partially inhibitory concentrations of KCN, cytochrome *c* in either the X- or the KCl extracted X-fragments showed uncoupler-sensitive, biphasic reduction kinetics upon the addition of NADH to the oligomycin-supplemented system. Under identical conditions rapid first order reduction kinetics were seen for cytochrome *c* in Y-fragments supplemented with either oligomycin or oligomycin + carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). (4) When cytochrome *c* was added to the mitochondrial suspension after sonication, a significant amount of cytochrome *c* was bound to both X- and Y-fragments, but was readily removed with a high ionic strength medium. (5) Lubrol had little effect on the ATPase activity of the X- and the Y-fragments, suggesting a lack of membrane-buried ATPase. (6) Partial depletion of ATPase in X-fragments did not induce an increase in reactivity towards externally added cytochrome *c*. (7) Both the X- and the Y-fragments showed an energy-linked fluorescence enhancement of 8-anilinonaphthalene-1-sulfonate and an energy-linked fluorescence decrease of quinacrine. (8) In the presence of K^+ nigericin alone or in combination with valinomycin exhibited a stimulating effect on the rate of NADH oxidase of the oligomycin-supplemented X- and Y-fragments.

Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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INTRODUCTION

In order to gain a closer insight into the precise role played by the apparent asymmetry of the mitochondrial inner membrane in respiratory chain-linked functions, especially those involved in energy coupling, we initiated studies [1] to fractionate and characterize membrane fragments derived from sonic disruption of beef heart mitochondria in the presence of EDTA. With a linear sucrose density gradient centrifugation, two distinct types of membrane fragments, designated as X and Y, have been obtained. The X-fragments consist of F_1-encrusted particles with diameters of 500–2000 Å, whereas the Y-fragments consist mainly of smooth particles with diameters of 300–900 Å. The X-fragments possessed a high energy coupling capacity, comparable to that exhibited by the conventional EDTA particles, as revealed from the oligomycin induced respiratory control and the energy-linked 8-anilinonaphthalene-1-sulfonate (ANS) fluorescence enhancement. A lower energy coupling capacity was exhibited by the Y-fragments. On the other hand, the Y-fragments exhibited a high reactivity towards externally added cytochrome *c* as shown by the high specific activity of NADH-cytochrome *c* reductase and cytochrome *c* oxidase. Under similar conditions a relatively low reactivity towards exogenous cytochrome *c* was exhibited by the X-fragments, similar to that observed with the conventional EDTA particles.

In this report we wish to present results of a study designed to characterize further these two types of membrane fragments. The study includes: (1) the binding behaviour and the kinetic pattern of reduction of both endogenous and exogenous cytochrome *c*, (2) the effect of Lubrol and cardiolipin on the ATPase, (3) the fluorescence responses of the extrinsic probes ANS and quinacrine and (4) the effect of ionophores such as nigericin and valinomycin in combination with K^+ on the NADH oxidase activity. Part of this work has been presented previously as a brief communication [2].

METHODS AND MATERIALS

The preparation of beef heart mitochondria and their sonicated suspension, the linear sucrose density gradient, and the procedure to fractionate and collect the gradient, were as described in the previous report [1]. In the present study, the correspondent membrane zones from 6 gradient tubes (39 ml in each tube) were collected and pooled. To avoid cross-contamination between the X and the Y zones, an intermediate zone between these two zones was collected which covered about 2–3 ml in each gradient tube. The pooled X-zone (about 60 ml) and Y-zone (30 ml) were then diluted with 0.5 volume of ice cold water and centrifuged at $105\,000 \times g$ for 65 min and 125 min, respectively. The respective pellet was then suspended in 0.25 M sucrose with a protein content of 10–20 mg/ml to form the preparation of the X-fragments and the Y-fragments in the present study.

The cytochrome content was estimated spectrophotometrically as described [1] previously at appropriate wavelength pairs with proper millimolar extinction coefficients, recorded by a Johnson Foundation Dual Wavelength Spectrophotometer or an Aminco DW-2 UV/VIS Spectrophotometer. The reduction kinetics of cytochromes were recorded in the same manner. The low temperature difference spectra of cytochromes were recorded with a wavelength scanning spectrophotometer as previously described [1].

The extraction of cytochrome *c* by 0.15 M KCl solution was performed essentially according to Jacobs and Sanadi [3]. The membrane fragments in 0.25 M sucrose solution were incubated with 0.15 M KCl at 0–4 °C for 10 min with constant stirring. The fragments were then centrifuged and resuspended in 0.25 M sucrose solution. This extraction was repeated twice.

NADH oxidase activity (NADH : O_2 oxidoreductase) was assayed spectrophotometrically by following the decrease in absorbance of NADH at 340 nm. The oligomycin induced respiratory control (R.C.I.) is defined as the ratio of the rate of NADH oxidase after and before the addition of FCCP to the oligomycin supplemented system. Protein was determined according to the procedure of Lowry et al. [4].

ATPase activity (ATP phosphorylase, EC 3.6.1.4) and 8-anilinonaphthalene-1-sulfonate (ANS) fluorescence measurements were as described previously [1]. The fluorescence measurement of quinacrine was performed with a Hitachi MPF-2A Spectrofluorometer using 420 nm for excitation and 500 nm for emission [5]. The extraction of ATPase with cardiolipin was performed essentially according to Toson et al. [6].

The total phospholipids was determined according to the procedure IB (10 % water in acetone containing ammonia) of Fleischer and Fleischer [7]. The determination of ubiquinone was according to Redfearn [8] except that pyrogallol was omitted.

Cytochrome *c* (Type VI from Horse heart), quinacrine, oligomycin and valinomycin were obtained from Sigma Chemical Company. Cardiolipin was obtained from Sylvania Company, Millburn, New Jersey. 8-Anilinonaphthalene-1-sulfonate (ANS) obtained commercially was purified as described previously. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift of Dr P. Heytler, E. I. Dupon de Nemours and Company. Nigericin was kindly supplied by Drs David Wong and J. M. McGuire of the Lilly Research Laboratories. All other chemicals are of the purest grade available commercially. Glass redistilled water was used throughout the present study.

RESULTS AND DISCUSSION

Contents of cytochromes, ubiquinone and total phospholipids

The contents of cytochromes, ubiquinone and the total phospholipids in the fractionated X- and Y-fragments and the conventional EDTA particle preparation are shown in Table I. These data indicated that on the protein basis the X-fragments possessed a relatively higher content with respect to the cytochromes and the total phospholipids. On the other hand, a relatively higher content of ubiquinone in the Y-fragments as compared with the X-fragments is noted. These results show that the difference between the two types of fragments with respect to functional and morphological properties is not reflected in their composition.

The proposal by Norling et al. [9] that the two *b* cytochromes, namely cytochrome b_{561} and cytochrome b_{566} might be functioning in two separate electron transport systems suggests that these cytochromes might be unevenly distributed between the X- and Y-fragments. Figs 1A and 1C show the difference spectra (reduced minus oxidized) for the X- and Y-fragments, respectively, with succinate as

TABLE I

CONTENTS OF CYTOCHROMES, UBIQUINONE AND TOTAL PHOSPHOLIPIDS

Preparation	nmol/mg protein				Phospholipids μg P/mg prot.
	a	$c + c_1$	*b*	Ubiquinone	
X	0.72	0.80	0.54	2.8	22.7
Y	0.55	0.64	0.35	3.5	19.5
EDTA-particles	0.64	0.77	0.51	2.8	18.1

the substrate in the presence of KCN. Figs 1B and 1D show the difference spectra (antimycin A + succinate + KCN minus succinate + KCN) resulting from antimycin A addition to the (succinate + KCN) pretreated systems for the X- and Y-fragments respectively. The ratios between cytochrome b_{561} and cytochrome b_{566} as estimated from these spectra are virtually identical for the X- and the Y-fragments. These data suggest that the two *b*-cytochromes are intimately associated with each other and the relatively low energy coupling capacity of the Y-fragments cannot result from a deficiency in the content of cytochrome b_{566}, although an impairment in its functional activity cannot be eliminated.

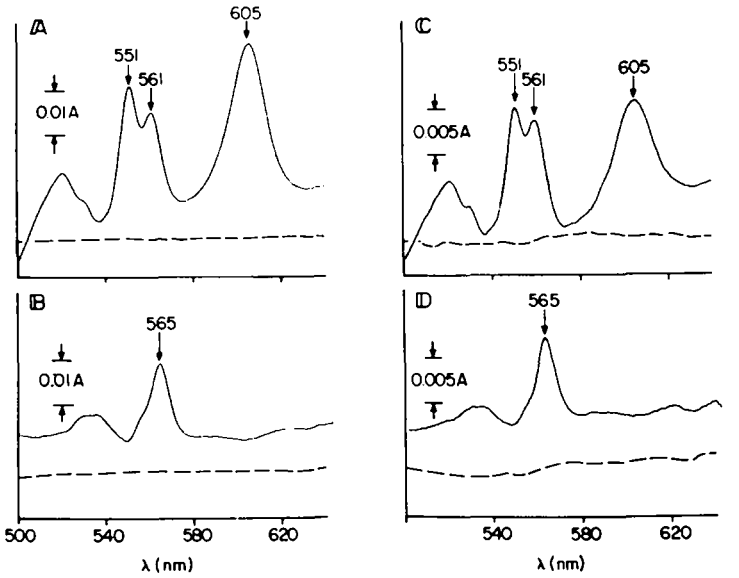


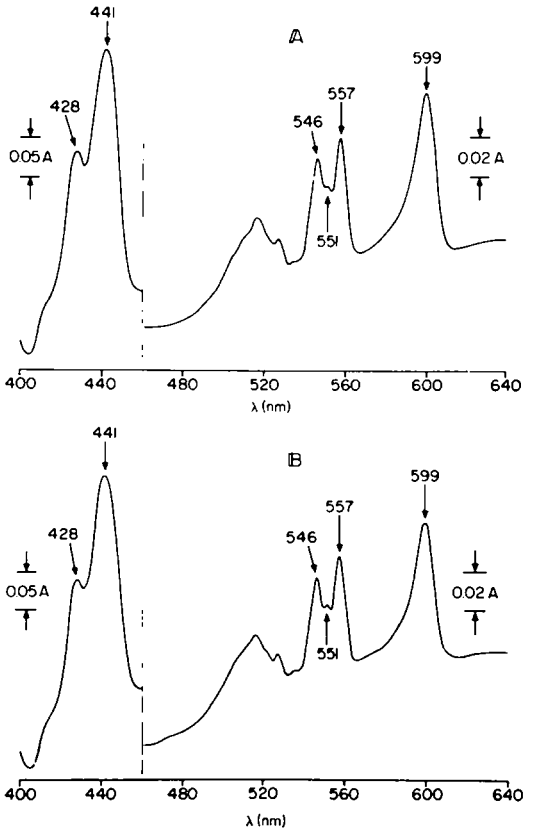
Fig. 1. Difference spectra of X- and Y-fragments reduced with succinate in the presence and absence of antimycin A. A and C: The reaction mixture consisted of 150 mM sucrose, 30 mM Tris-acetate, pH 7.5, 2 mM KCN, and 10.2 mg protein of X-fragments (A) or 12.4 mg protein of Y-fragments (C), total volume, 6.0 ml; 3.0 ml of the suspension was then placed in the measuring cell, and the remainder in the reference cell. A base line (dotted line) was recorded. Difference spectra were then recorded 3 min after the addition of 5 mM succinate to the measuring cell. B and D: Conditions were the same as in A and C except that 5 mM succinate was in both the measuring and reference cell. The dotted lines were the base line. Difference spectra were recorded 2 min after the addition of 5 μg antimycin A into the measuring cell. Temperature: 25 °C.

TABLE II

EFFECT OF KCl TREATMENT ON ENDOGENOUS CYTOCHROME *c* OF X- AND Y-FRAGMENTS

For NADH oxidase, the reaction mixture consisted of 150 mM sucrose, 30 mM Tris-acetate, pH 7.5, and 0.23 mg, 0.18 mg, 0.31 mg and 0.24 mg protein of X-, KCl-treated X-, Y- and KCl treated Y-fragments, respectively. Total volume, 3 ml; temperature, 28 °C. When indicated, 3.3 μM cytochrome *c* was also present. Others were as described in Methods and Materials.

	c + c_1		*a*		NADH oxidase nmol/min/mg protein	
	nmol/mg protein	% of total	nmol/mg protein	% of total	−Cyt. *c*	+Cyt. *c*
X	0.61	(100)	0.55	(100)	660	705
Extracts		7.3	–	0.4	--	--
KCl-treated X	0.58	76.9	0.54	77.7	680	750
Y	0.55	(100)	0.54	(100)	407	750
Extracts		29.8		0.4		
KCl-treated Y	0.42	59.7	0.46	65.7	332	987



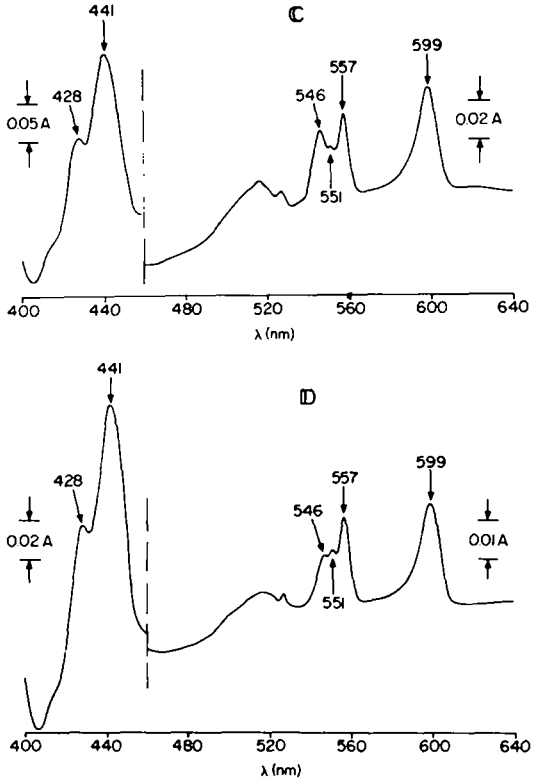


Fig. 2. Low temperature (77 °K) difference spectra of the X- and Y-fragments before and after KCl extraction. The reaction mixture consisted of 150 mM sucrose, 30 mM Tris-acetate, pH 7.5, and various amounts of protein of each fragment preparation as indicated below. Difference spectra were recorded 2 min after the addition of 1 mM NADH to the measuring cell. 2.9 mg/ml and 3.8 mg/ml of X- and KCl extracted X-fragments were present for spectra A and B, respectively; 2.8 mg/ml and 1.4 mg/ml of Y- and KCl extracted Y-fragments were present for spectra C and D, respectively.

Effect of KCl treatment

As reported previously [1], the Y- but not the X-fragments were highly reactive towards externally added cytochrome *c*. Yet, the *c*-cytochrome content of the Y-fragments (Table I) was as high as 80 % of that of the X-fragments. It is then of importance to assess the effect of KCl treatment on the endogenous cytochrome *c* of the X- and the Y-fragments, since KCl at 0.15 M has been shown to extract more than 95 % of the total cytochrome *c* in swollen mitochondria [3]. Table II shows the effect of KCl treatment on the cytochrome *c* content of X- and Y-fragments. The *c*-cytochrome content was estimated by measurement of absorbance difference (reduced minus oxidized) at the wavelength pair 550 minus 540 nm which actually reflects the total contribution from both cytochrome *c* and cytochrome c_1 in the membrane. Only cytochrome *c* is extractible by KCl solution. The supernatant from the two KCl treatments of the X-fragments contained an amount of cytochromes equivalent to 7 % of the *c*-cytochromes and 0.4 % of the *a* cytochromes, whereas the supernatant from the Y-fragments contained 30 % of the *c* cytochromes and 0.4 % of the *a*

cytochromes. These results clearly indicate a favorable extraction of cytochrome *c* from the Y-fragments. This conclusion is further supported by the low temperature difference spectra shown in Fig. 2. The spectra of the X- (Fig. 2A) and the KCl-treated X- (Fig. 2B) fragments are virtually identical. This is in line with the data in Table II showing that the endogenous cytochrome *c* in the X-fragments is virtually non-extractable. The spectrum of the untreated Y-fragments (Fig. 2C) is similar to that of the X-fragments (cf. Fig. 2A). However, in the spectrum of the KCl-treated Y-fragments (Fig. 2D), the peak at 546 nm, which is contributed mainly by cytochrome *c*, is greatly diminished, whereas other major peaks in this spectrum are not significantly altered when compared to those of the untreated Y-fragments (cf Fig. 2C). These data further substantiate our earlier conclusion that a significant amount of cytochrome *c* in the Y-fragments can be removed by direct KCl treatment. The partial removal of endogenous cytochrome *c* in the Y-fragments by KCl is also reflected in the decrease in rate of NADH oxidase activity (Table II). Addition of cytochrome *c* to the KCl-treated Y-fragments stimulated the rate of NADH oxidase to a level which is even higher than that exhibited by the untreated fragments (Table II). In line with our previous observations [1], externally added cytochrome *c* exhibits very little effect on the NADH oxidase activity of the X-fragments, either before or after KCl treatment.

The relatively low extractibility of cytochrome *c* in the X-fragments, which possess little reactivity towards externally added cytochrome *c*, is consistent with the idea that the cytochrome *c* molecules may be enclosed inside the fragments. These properties are in contrast to those of swollen mitochondria [3, 10] and the Y-fragments where cytochrome *c* may be located in a region of the membrane which is readily equilibrating with external medium and exhibits a high degree of reactivity towards exogenous cytochrome *c*. These data clearly indicate a close correlation between the degree of extractibility of the endogenous cytochrome *c* and the degree of reactivity towards exogenous cytochrome *c*.

Binding of externally added cytochrome c

Previous studies by Lee [11] on conventional EDTA-particle preparations established that, if external cytochrome *c* was added to mitochondrial suspension prior to sonication, the resultant EDTA-particle preparation increased its cytochrome *c* content up to 5 times. The loaded cytochrome *c* was not extractible with a high ionic strength medium and behaved like endogenous cytochrome *c* in reduction kinetics. When added after sonication, cytochrome *c* was also significantly loaded into the particle preparation but was readily washed out with a high ionic strength medium. Similar experiments were performed with the fractionated X- and Y-fragments. When cytochrome *c* was added before sonication, both the X- and the Y-fragments picked up significant amounts of cytochrome *c*. A 6–8-fold increase in cytochrome *c* content of both X- and Y-fragments was noted. After two KCl treatments, the X-fragments retained 2/3 of total cytochrome *c*, whereas the Y-fragments retained 1/4. These data indicated that in the X-fragments, most of the added cytochrome *c* was bound in a manner inaccessible to KCl treatment, similar to that observed in the EDTA-particles. On the other hand, most of the cytochrome *c* bound to the Y-fragments was readily extractible by merely increasing the ionic strength of the medium.

When cytochrome *c* was added to the mitochondrial suspension after sonication, the cytochrome *c* content of the X-fragments increased by a factor of 3–4, whereas that of the Y-fragments increased by a factor of 7–9. Two KCl treatments caused the X-fragments to lose about 60 % and the Y-fragments about 90 % of the total cytochrome *c*. These data are in line with those obtained with EDTA-particles; cytochrome *c* molecules loaded under these conditions appear to be bound to the surface of the membrane and are readily dissociated by a high ionic strength medium.

TABLE III

NADH OXIDASE ACTIVITY OF THE CYTOCHROME *c* ENRICHED Y-FRAGMENTS

The loadings of the externally added cytochrome *c* into the membrane fragments were performed by incubating 26 mg cytochrome *c* with 40 ml of mitochondrial suspension (containing 422–460 mg protein) after sonic disruption. For NADH oxidase, the reaction mixture consisted of 150 mM sucrose, 30 mM Tris-acetate, pH 7.5, and 0.08 to 0.12 mg protein. The reaction was initiated upon the addition of 200 μM NADH. Total volume, 2.5 ml; temperature, 26 °C. Others when indicated were: 5 μg oligomycin, 2 μM FCCP and 4 μM cytochrome *c*.

	c	NADH oxidase (nmol/min/mg protein)			Respiratory control index
		− oligo	+ oligo	+ oligo + FCCP	
Y	−	809	707	829	1.2
	+	917	897	975	1.1
KCl treated Y	−	183	143	190	1.3
	+	878	795	811	1.0

As shown in Table III, the cytochrome *c* loaded Y-fragments derived from incubation of cytochrome *c* with the sonicated mitochondrial suspension exhibited a relatively high NADH oxidase activity, comparable to that of the unloaded Y-fragments in the presence of optimal amount of externally added cytochrome *c* [1]. Externally added cytochrome *c* gave only a slight stimulatory effect on the NADH oxidase activity of the cytochrome *c* loaded Y-fragments. Two KCl treatments caused a loss of approximately 90 % of the total cytochrome *c*. The KCl-treated Y-fragments exhibited a low NADH oxidase activity which could be stimulated 4-fold by externally added cytochrome *c*. Under similar conditions, externally added cytochrome *c* did not alter the NADH oxidase activity of the untreated and the KCl-treated X-fragments (not shown). These results indicate that the externally added cytochrome *c* which served to stimulate the NADH oxidase activity in the Y-fragments was actually bound to the fragments, rather than acting as a soluble substrate in the medium, as Camerino and Smith have proposed [12] for the externally added cytochrome *c* which forms a bypass around the endogenous cytochrome *c* of heart-muscle particles.

Reduction kinetics of cytochrome c

It has been reported [13, 14] that when partially inhibitory concentrations of KCN are added to the EDTA particles supplemented with oligomycin and NADH or

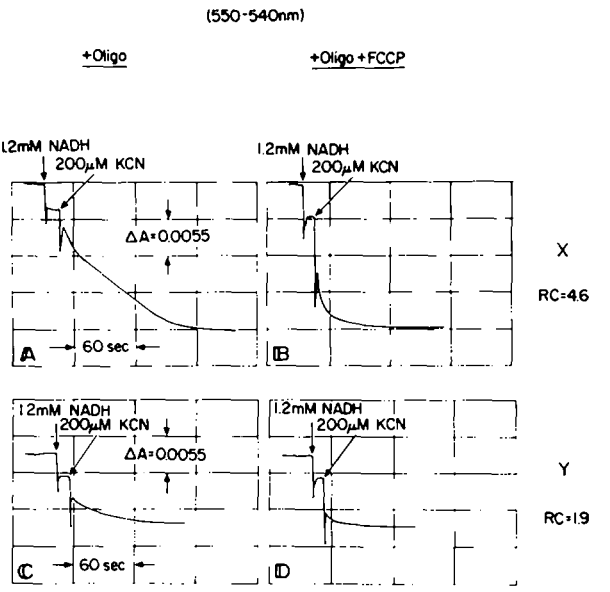


Fig. 3. Reduction kinetics of endogenous cytochrome *c*. The reaction mixture consisted of 150 mM sucrose, 30 mM Tris-acetate, pH 7.5 and 2.8 mg protein of X-fragments (A and B) or 3.1 mg protein of Y-fragments (C and D). Total volume, 2.5 ml, temperature, 25 °C. Others when indicated were: NADH, 1.2 mM, KCN, 200 μM, oligomycin, 5 μg and FCCP, 2 μM.

succinate, the reduction kinetics of cytochromes *c* and *a* are biphasic: a relatively rapid initial phase followed by a slow phase. The slow phase can be abolished by the addition of uncouplers. These biphasic kinetics, together with the residual oligomycin-resistant respiration in EDTA particles raised the question [13, 14] as to whether heterogeneity in population of fragments and/or in respiratory chain assemblies did occur and did play an important role in the metabolic properties of EDTA particles. The possible role of heterogeniety in the population of EDTA particles was therefore evaluated with regard to cytochrome reduction kinetics, using the purified fraction, such as X-fragments.

The reduction kinetics of cytochrome *c* with NADH as substrate under various metabolic states are shown in Fig. 3. In the presence of oligomycin and NADH, upon the addition of 200 μM KCN, the X-fragments exhibited a rapid initial phase and a subsequent slow phase of reduction. The slow phase can be abolished completely upon the addition of FCCP (Fig. 3B). Similar results were also obtained for cytochrome *a* (not shown). The similarity of the kinetic behaviours of cytochromes *c* and *a* and various other biochemical properties [1] exhibited by X-fragments as compared with those exhibited by the conventional EDTA particles indicate that the oligomycin-induced control of electron transport of EDTA particles does not originate from the heterogeneity in population of membrane fragments. These observations together with the distinct difference in activation energy of NADH oxidase in the uncoupled and the oligomycin-coupled states of EDTA particles [15] support the concept that oligomycin exerts a multisite control of the respiratory chain. The oligomycin-resistant respiration and the oligomycin-induced biphasic

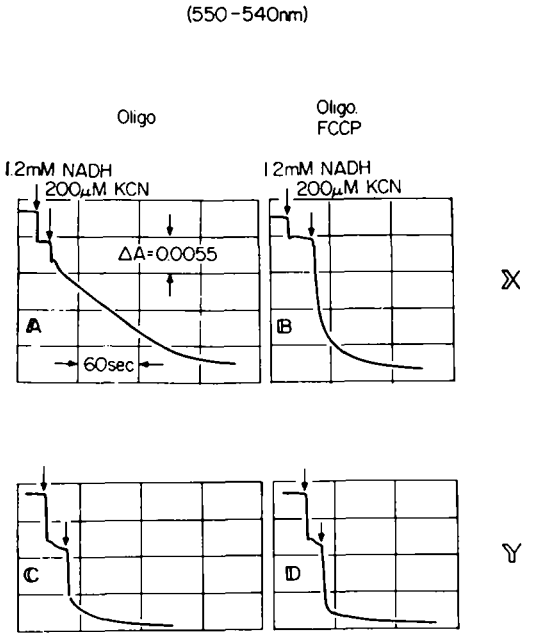


Fig. 4. Reduction kinetics of cytochrome *c* of fragments derived from mitochondrial suspension treated with cytochrome *c* before sonic disruption. Conditions were as in Fig. 3 except that the membrane fragments were derived from mitochondrial suspension (248 mg protein) which has been pretreated with 13 mg of cytochrome *c* prior to sonic disruption. 0.76 mg protein of X-fragments were employed for A and B; and 0.78 mg protein of Y-fragments were employed for C and D, respectively. Others were as indicated.

reduction kinetics of the respiratory chain carriers thus appear to be intrinsic properties of the respiratory chain-linked energy coupling.

As would be expected, the Y-fragments, which possessed a low oligomycin-induced respiratory control, did not show distinctive biphasic reduction kinetics of cytochromes *c* and *a* in the oligomycin supplemented system (Figs 3C and 3D).

Fig. 4 shows the reduction kinetics of the cytochrome *c* incorporated into the X- and Y-fragments derived from incubation of cytochrome *c* with the mitochondrial suspension prior to sonication. The kinetic behaviour of the incorporated cytochrome *c* in the X- and Y-fragments was virtually identical to that of the endogenous cytochrome *c* of X- and Y-fragments respectively. These observations together with the binding properties of added cytochrome *c* in the X-fragments substantiate the conclusion [11] drawn from studies on EDTA particles that only the cytochrome *c* molecules incorporated into the X-fragments behaved kinetically similar to endogenous cytochrome *c*.

Membrane ATPase subunits or F_1

The location of the membrane ATPase (F_1) has been used as a morphological marker for the orientation or sideness of the mitochondrial membrane [16]. If an atractyloside sensitive translocating system is needed to transport adenine nucleotides across the membrane, as was demonstrated in intact mitochondria [17], the existence

TABLE IV

EFFECT OF LUBROL ON ATPase ACTIVITY (μmol P_i/min/mg protein)

The reaction mixture consisted of 200 mM sucrose, 50 mM Tris · HCl, pH 7.5, 4 mM $MgSO_4$ and varying amounts of protein (0.15 mg for X- and 0.36 for Y-fragments in Experiment 1 and 0.23 mg for X- and 0.41 mg for Y-fragments in Experiment 2). The reaction was started upon the addition of 5 mM ATP. Total volume, 1 ml. The reaction was terminated by the addition of 1 ml of 20% perchloric acid after 5 min of incubation at 30 °C. When indicated, 5 μg oligomycin was added.

Expt.	Preparation	Lubrol (mg/assay)				
		0	0.5	1	2	4
1	X, − oligomycin	2.2	2.1	2.1		
	X, + oligomycin	0	–	0.1	–	–
	Y, − oligomycin	1.1		1.0	1.0	–
	Y, + oligomycin	0.04	–		0.2	–
2	X, − oligomycin	1.7	–	1.7	1.6	1.5
	Y, − oligomycin	1.0		–	0.9	1.0

of a membrane barrier to ATP for ATPase activity would depend on where the ATPase is located. We reported previously [1] that the low ATPase activity in the Y-fragments was insensitive to atractyloside. This seemed to exclude the possible localization of ATPase inside the Y-fragments. However, we were not able to eliminate the possibility that there might be some ATPase located inside the Y-fragments which was unable to utilize the added ATP because of a lack of an operational translocase system, or that the membrane is permeable to ATP. The similarity in cytochrome *c* reactivity between the Y-fragments and the swollen mitochondria suggests the need to test whether there is ATPase buried inside the Y-fragments. Table IV shows the ATPase activity of the X- and the Y-fragments measured in the presence of a non-ionic detergent, Lubrol, which presumably breaks up the membrane. The results show that Lubrol did not affect the ATPase activity of either the X- or the Y-fragments. The ineffectiveness of Lubrol suggests that all the functional ATPase molecules were accessible to added ATP. It is interesting to note that Lubrol did not abolish the oligomycin sensitivity of the ATPase.

Failure to disclose any membrane-buried ATPase activity in the Y-fragments prompted us to investigate the effect of depletion of ATPase from the X-fragments, since it was possible that the Y-fragments might resemble the X-fragments but be deficient in ATPase. Cardiolipin has been reported to remove a portion of ATPase from beef heart submitochondrial particles [6, 18]. As shown in Table V, if cardiolipin was used to reduce the specific activity of ATPase of the X-fragments from 2.10 to 0.77 μmoles P_i released/min/mg protein (Table V A), did not develop any additional cytochrome *c* reactivity, e.g. the addition of cytochrome *c* did not stimulate the NADH oxidase activity (Table V B). Thus, in this respect, the partially ATPase-depleted X-fragments appeared to be different from the Y-fragments.

Fluorescence change of ANS and quinacrine

The energy-dependent fluorescence change of fluorescent probes has been employed as one of the convenient approaches to study the energized state of the

TABLE V

EFFECT OF EXTRACTION BY CARDIOLIPIN ON ACTIVITIES OF ATPase AND NADH OXIDASE IN THE X-FRAGMENTS

For ATPase, conditions were as described in Table IV except that 0.19 mg and 0.15 mg protein for the X- and the cardiolipin treated X-fragments, respectively, were used. In the case of the supernatant the reaction was initiated upon the addition of 100 μl of preincubated (at 30 °C) supernatant instead of ATP. For NADH oxidase, the reaction mixture consisted of 150 mM sucrose, 30 mM Tris-acetate, pH 7.5, and 0.15 to 0.30 mg protein. The reaction was initiated upon the addition of 200 μM NADH. Others when indicated were: 5 μg oligomycin, 2 μM FCCP and 4 μM cytochrome *c*.

A. ATPase activity

	μmol P_i/min/mg protein			
	Particles		Supernatant	
	−oligomycin	+oligomycin	−oligomycin	+oligomycin
X	2.10	0.05	0.36	0.52
Cardiolipin treated X	0.77	0.67	8.03	7.46

B. NADH oxidase activity

	c	nmol NADH/min/mg protein			Respiratory control
			−oligomycin	+oligomycin + FCCP	
X	-	731	200	796	4.0
	·	832	260	944	3.6
Cardiolipin treated X	·	597	196	555	2.8
	·	591	196	547	2.8
Y	--	407	249	589	2.4
	+	751	629	957	1.5

membrane. ANS has been shown [19–21] to exhibit a fluorescence enhancement in EDTA-particle preparation upon energization. Less well established is the observation [22] that in intact mitochondria, energization caused a fluorescence decrease of ANS. The opposite directions of the ANS fluorescence change in intact mitochondria and the submitochondrial particles had led several investigators [22, 23] to postulate that ANS could be used as a probe for membrane orientation, despite the fact that the rate of ANS fluorescence change in mitochondria was very low as compared to that observed in submitochondrial particles. Another fluorescence probe, quinacrine, has recently been shown [5] in EDTA-particles to exhibit an energy-dependent decrease of fluorescence. Previous work [1, 2] has established that the X-fragments exhibited the highest oligomycin-induced ANS fluorescence increase. As can be seen from Fig. 5, the traces of the ANS fluorescence response (on the left side) showed that the Y-fragments exhibited a fluorescence increase similar to that observed in the X-fragments, although at a smaller magnitude. The traces on the right side are the fluorescence response of quinacrine in oligomycin-supplemented X- and Y-fragments in the

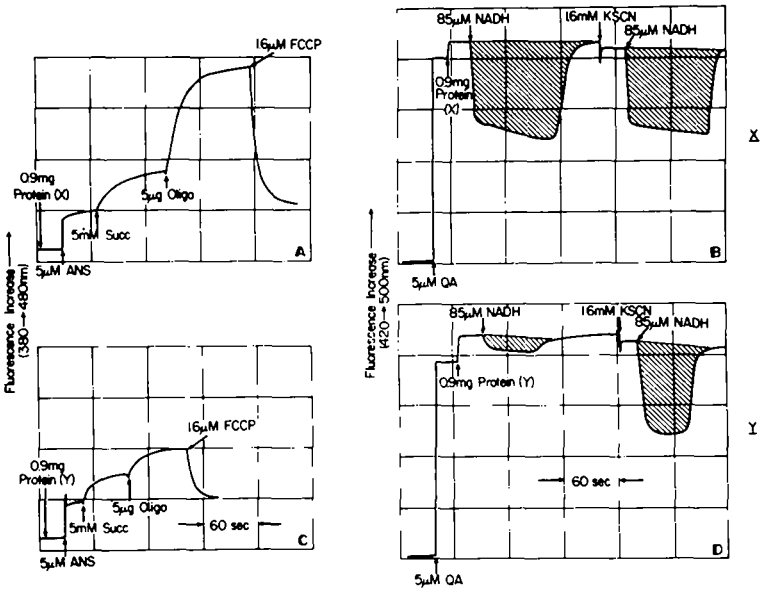


Fig. 5. Fluorescence responses of ANS and quinacrine. The reaction mixture consisted of 150 mM sucrose, 30 mM Tris-sulfate, pH 7.5, and 0.9 mg protein of either X- or Y-fragments. Total volume, 3 ml; temperature, 21 °C. Others were as indicated.

absence and presence of KSCN. Both the X- and the Y-fragments showed an energy-linked fluorescence decrease. In the absence of KSCN, the extent of decrease was higher in the X-fragments than in the Y-fragments. Yet, in the presence of KSCN, the extents of decrease in both the X- and the Y-fragments were almost equal. Therefore, although some quantitative difference is seen under certain conditions, no difference in the direction of fluorescence change of ANS and quinacrine between the X- and the Y-fragments can be detected.

Effect of ionophores

In the presence of K^+, a combination of two lipid-soluble ionophores, nigericin and valinomycin, has been shown to release the oligomycin-induced respiratory control in EDTA-particles [24–26]. This is presumably due to the energy consumption because of a cyclic influx and efflux of K^+ induced respectively by nigericin and valinomycin [25]. The ionophore-induced movement of K^+ has been considered as vectorial; its direction depends on the membrane polarity [26]. Hence, it is of interest to see the effect of these ionophores in the presence of K^+, on the oligomycin-induced respiratory control of the X- and the Y-fragments. The effect of nigericin and valinomycin at low (0.3 μg) and high (3 μg) concentrations in the presence of 16.7 mM KCl, on the NADH oxidase activity is shown in Fig. 6. In both the X- and the Y-fragments, the presence of KCl at this concentration caused an increase in both the oxidase activity and in the respiratory control index. In this experiment, with FCCP as the uncoupler, the index of the X-fragments was slightly increased by KCl from 3.6 to 4.6 and the Y-fragments from 2.8 to 3.6. It is clear from Fig. 6 that in both the X- and the Y-fragments, nigericin alone only partially released the control, whereas

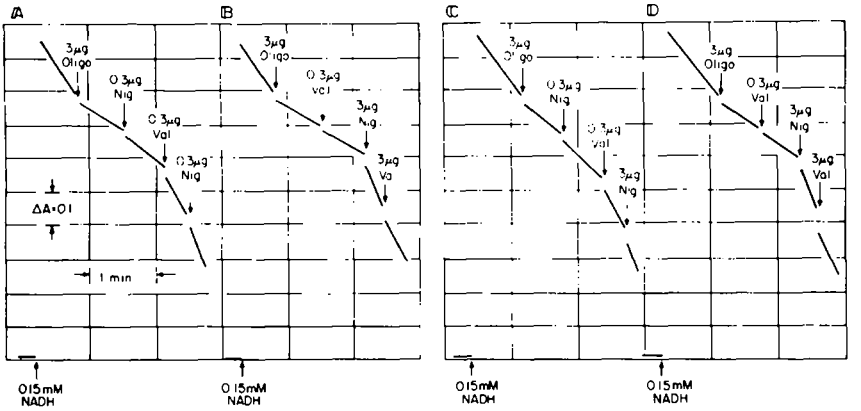


Fig. 6. Effects of nigericin and valinomycin on NADH oxidase. The reaction mixture consisted of 150 mM sucrose, 16.7 mM KCl, 30 mM Tris-acetate, pH 7.5, and 0.21 mg protein of X-fragments (A, B) or 0.31 mg protein of Y-fragments (C, D). Total volume, 3 ml; temperature, 26 °C. The reaction was initiated upon the addition of 200 μM NADH. Others were as indicated.

the valinomycin alone had little effect. A combination of both ionophores effectively released the control. These properties are similar to those reported by Montal et al. [25] with the conventional EDTA submitochondrial particles. Thus, no obvious qualitative difference in the effect of ionophores can be seen between the X- and the Y-fragments.

The similarity in the effect of ionophores was further substantiated by the results from Fig. 7 which shows the titration profile of the nigericin against % stimulation of the oligomycin-coupled NADH oxidase activity in the presence and absense of valinomycin. The limited extent of release for nigericin alone reached a plateau at about 0.5 μg nigericin per mg protein for both the X- and the Y-fragments. In the presence of valinomycin (1.4 μg/mg protein for X-fragments and 1.0 for Y) the

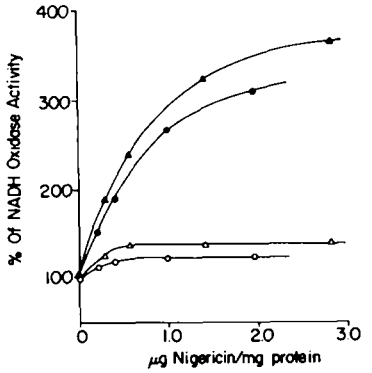


Fig. 7. Titration of nigericin on the release of the oligomycin induced respiratory control of NADH oxidase in the absence and presence of valinomycin. Conditions were as in Fig. 6 except that varying amounts of nigericin were used. For X-fragments: -△-, nigericin alone, -▲-, nigericin + valinomycin (1.5 μg/mg protein). For Y-fragments: -○-, nigericin alone and -●-, nigericin + valinomycin (1.0 μg/mg protein).

amount of nigericin needed to reach 50 % of the maximal release by FCCP was about 0.8 μg/mg protein for the X-fragments and 0.7 μg/mg for the Y. From these studies it is concluded that no significant difference between the X- and the Y-fragments in the properties of the ionophore-mediated ion uptake can be observed.

CONCLUSION

Data presented in this study along with results reported in the previous paper of this series, clearly demonstrate that, among the membrane fragments obtained by the sucrose density gradient, the X-fragments are the most active component with respect to the functional activities related to mitochondrial energy metabolism. Morphologically, these fragments follow the description for the "inside-out" particles described by Lee and Ernster [27], although it is still not clear whether there is any aqueous space enclosed inside the X-fragments. Although accounting for only 1/3 of the total protein in EDTA particle preparation, the X-fragments have been shown to be the component responsible for most of the functional characteristics of the energy metabolism in EDTA particle preparation. Therefore, it is reasonable to use the EDTA particle preparation "as is" for studies of the respiratory chain-linked energy transfer reactions. However, for precise studies of the structure-function relationships of the mitochondrial inner membrane, the X-fragments, which possess better defined morphological characteristics, are the preparation of choice.

The Y-fragments, although poor in the energy-linked functional activities, exhibited a high reactivity towards externally added cytochrome *c*. Morphologically these fragments have relatively smooth surface. On the basis of the relatively ready extractibility of the endogenous cytochrome *c* and of the binding behaviour of the externally added cytochrome *c*, one might propose that the membrane orientation of the Y-fragments may be opposite to that of the X-fragments but similar to that of the swollen mitochondria [3, 10]. However, the failure of atractyloside to inhibit and of Lubrol to stimulate the ATPase activity of Y-fragments would argue against this proposal, although the possibility of loss of the adenylate translocase and modification of the membrane permeability towards ATP during the preparation cannot be eliminated. It is possible that the different responses of cytochrome *c* in various kinds of membrane fragments may simply reflect a different extent of shielding of the reactive sites of cytochrome *c* from the medium by a barrier, resulting from the action [14] of scrambling, dislocation or others due to the sonic disruption, other than the inversion of the whole membrane assembly.

The similarity in the responses of the X- and Y-fragments revealed by the fluorescence probes, ANS and quinacrine, in various metabolic states has interesting implications concerning the membrane orientation of the X- and Y-fragments. Our previous work strongly suggests that the probes ANS and quinacrine report two aspects of the energized state of the membrane: change in the conformation and change of a hydrophobic environment in the membrane in the case of ANS and local proton binding in the membrane in the case of quinacrine [28]. If these two aspects of the energized state depend on membrane orientation, as has been suggested [22, 23], then the X- and Y-fragments have the same orientation and are both capable of energy coupling responses, yet the Y-fragment membrane is relatively more permeable to cytochrome *c*. If one postulates the "inside-out" orientation for the X- and the "right-

side out" for the Y-fragments to explain cytochrome *c* extractibility, then one must also postulate that the energy coupling responses of the membrane probes are independent of membrane orientation. If one accepts the latter postulate, it is intramembrane reactions which are of importance; transmembrane reactions are irrelevant in this case. Precisely the same set of arguments applies to the responses of the X- and Y-fragments to ion movements mediated by ionophores. These results imply that either the membrane in submitochondrial particles capable of energy coupling is permeable to cytochrome *c* under the proper conditions or that membrane orientation is irrelevant to energy-coupling reactions. In either case, arguments utilizing the sidedness of submitochondrial particles to confirm or reject hypotheses of energy conservations must henceforth be viewed with extreme caution.

During the preparation of this report, a paper by Astle and Cooper [29] came to our attention. When they subjected the submitochondrial particles derived by sonication of rat liver inner membrane matrix complex to Ludox gradient centrifugation, they obtained two major bands. Based on the labelling pattern with iodide catalyzed by lactoperoxidase they have concluded that the particles in the bottom band were "inside-out" and those in the top band were "right-side out". Both bands were capable of oxidative phosphorylation. However, the phosphorylating activity of both bands was relatively low (12–17 nmol P_i uptake/min/mg protein) as compared to that of the starting preparation (290 nmol P_i uptake/min/mg protein). The residual phosphorylating activity of both bands was sensitive to oligomycin, but insensitive to atractyloside. It should be pointed out that the particle preparation used by Astle and Cooper [29] is not identical to the EDTA particle preparation employed by us. Nevertheless, based solely on the sequence of the sedimentation, one might be tempted to equate the top and the bottom band of their gradient to our Y- and X-band respectively. Unfortunately, Astle and Cooper [29] did not report studies in detail on functional activities of their bands other than the phosphorylating activity, which was rather low (approximately 5 % of the starting preparation). Hence, it is not possible at this time to construct a meaningful comparison between their and our preparations.

ACKNOWLEDGEMENTS

This work has been supported by research grants from the National Institutes of Health, GM 19636, and the Muscular Dystrophy Associations of America, Inc. The authors wish to thank Dr B. T. Storey for stimulating discussions and Ms B. I. T. Cierkosz for valuable technical assistance.

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