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CROSS-LINKING OF MITOCHONDRIAL MATRIX PROTEINS IN SITU

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Different cross-linkers (10 mM) of varying specificity and arm length were found to cross-link mitochondrial matrix proteins in situ in 2 min at pH 7.4. As seen by SDS-polyacrylamide electrophoresis, the disappearance of individual protein bands was accompanied by concomitant appearance of polymeric aggregates that failed to enter the 4% spacer gel. The disorganization of the mitochondrial matrix infrastructure either by swelling or sonication of the mitochondria resulted in a decrease in the rate of cross-linking. Leakage of citrate synthase, malate dehydrogenase and fumarase was found to be reduced when cross-linked mitochondria were made permeable with toluene. On lysing the cross-linked mitochondria, a major part of the matrix protein (75%) was found to sediment with the membrane fraction. The activities of citrate synthase, malate dehydrogenase and fumarase in rat liver mitochondria were also found to increase in the precipitates with a concomitant decrease in their activities in the soluble matrix fraction. These results indicate that the cross-linker enters the mitochondria and cross-links matrix proteins including Krebs cycle enzymes either to the mitochondrial membranes, or to themselves resulting in very large molecular weight complexes. These results are interpreted to mean that in liver mitochondria, the Krebs cycle enzymes are preferentially located near the membrane.

Introduction

The quaternary structure of oligomeric proteins and protein-protein associations in membranes and the structure of ribosomes have all been studied using the technique of chemical cross-linking [1]. The specific compartmentation of enzymes in cells and cellular organelles [2,3] has also been confirmed using techniques involving cross-linking by glutaraldehyde. A few reports have appeared on the effects of cross-linking of mitochondrial membranes [4–7]. Tinberg et al. [4] have studied the possible involvement of molecular motions in electron transport using dimethyl suberimidate dihydrochloride cross-linked mitochondria. Tinberg et al. [5] have also shown that molecular cross-linking of isolated mitochondrial membranes with dimethyl suberimidate dihydrochloride results in inhibition of electron transport and ATPase activity. Rendon et al. [6] have studied the proton permeation, adenine nucleotide translocation and respiratory control in mitochondria cross-linked with dimethyl suberimidate dihydrochloride. Rendon and Waksman [7], using this technique, have demonstrated that organizational changes of mitochondrial membranes induced by succinate, including intramitochondrial protein movement, can be prevented by carbonyl cyanide *p*-trifluorometho-

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

xyphenylhydrazone, an uncoupler of oxidative phosphorylation. Even though these studies have indicated that cross-linking mitochondrial membranes using dimethyl suberimidate dihydrochloride can modify certain membrane properties, little information is available concerning the effect of cross-linkers on the matrix proteins of cross-linked intact mitochondria.

It was generally assumed for many years that the Krebs cycle enzymes are randomly distributed in the mitochondrial matrix, but recent studies have predicted the organization of these enzymes as a multienzyme complex on or off the mitochondrial inner membrane [8–11]. We have used a variety of cross-linking reagents on intact mitochondria and have studied their effect on the matrix proteins and more specifically, their effect on the Krebs cycle enzymes, citrate synthase, malate dehydrogenase and fumarase. We conclude that Krebs cycle enzymes have a preferential location within the matrix of liver mitochondria next to the inner membrane.

Materials and Methods

The following chemicals were obtained from the indicated sources: bovine serum albumin, Lubrol WX, glutaraldehyde (25% solution), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, hydrazine, NADH and DTNB (Sigma Chemical Co., St. Louis, MO); digitonin and oxalacetic acid (Calbiochem, La Jolla, CA); Fluram (Roche Diagnostics, Nutley, NJ); Nagarse (Enzyme Development Corp., Penn Plaza, NY); methyl acetimidate hydrochloride, dimethyl suberimidate dihydrochloride, dimethyl adipimidate dihydrochloride, disuccinimidyl tartarate, 1,5-difluoro-2,4-dinitrobenzene (Pierce Rockford, IL); acrylamide (Eastman Kodak, Rochester, NY); *N,N'*-methylenebisacrylamide and *N,N,N'N'*-tetramethylethylenediamine (Miles Laboratory Inc., Elkhart, IN); slab gel apparatus, plates and molecular weight protein markers (Bio-Rad, Richmond, CA); SDS and polyacrylamide ($M_r > 5 \cdot 10^6$) (BDH Ltd., Poole U.K.); CoA (P.L. Biochemicals, Milwaukee, WI); polyethylene glycol (M_r 6000–7500) (Matheson, Coleman and Bell Co., Norwood, OH); CoA-Ac was prepared according to a previously described method [12].

Isolation of mitochondria and mitochondrial subfractions

Mitochondria were isolated from livers and hearts of fasted (24 h) male Sprague-Dawley rats by the methods described earlier [13–15]. Isolation medium without bovine serum albumin was used in the final wash. Mitochondria were made permeable using toluene as described by Matlib et al. [16]. Swelling of mitochondria was carried out as described by Matlib and Srere [14]. Mitoplasts, outer membranes and intermembrane space proteins were obtained from rat liver mitochondria using digitonin [17]. The mitochondrial inner membranes were obtained by lysing the mitoplasts using Lubrol WX [17]. Matrix proteins were obtained by lysing the mitoplasts by repeated freeze-thawing followed by centrifugation at $144\,000 \times g$ for 60 min. The purity of these preparations has been established previously [18] and has been routinely monitored for the preparations used here.

Cross-linking reactions

Glutaraldehyde. Glutaraldehyde was diluted to 2% in a medium containing 220 mM mannitol, 70 mM sucrose, 50 mM Hepes (pH 7.4). Appropriate aliquots were added to the mitochondrial suspension (2 mg/ml) in the above buffer, to give final glutaraldehyde concentrations of 0.02–0.1% and incubated at 25°C. A stock solution of 0.5 M hydrazine adjusted to pH 7.4 was used as a quencher for the cross-linking reaction. To arrest cross-linking, a 5-fold molar excess of the quencher relative to initial glutaraldehyde concentration was added.

Imidates. Mitochondria (2 mg/ml) were suspended in a medium containing 220 mM mannitol, 70 mM sucrose and 50 mM Hepes (pH 7.4) (cross-linking medium). Stock imidate solutions (methyl acetimidate hydrochloride, dimethyl suberimidate dihydrochloride, dimethyl adipimidate dihydrochloride, dimethyl pimelimidate dihydrochloride, disuccinimidyl tartarate) were made in the above buffer just before use and adjusted to pH 7.4. Appropriate aliquots to give final concentrations of 10 mM (dimethyl suberimidate dihydrochloride, dimethyl adipimidate dihydrochloride, dimethyl pimelimidate dihydrochloride, disuccinimidyl tartarate), or 20 mM (methyl acetimidate hydrochloride) were added to a

mitochondrial suspension and incubated at 25°C. To arrest cross-linking, a 10-fold molar excess of ammonium acetate relative to initial imidate concentration was added.

1,5-Difluoro-2,4-dinitrobenzene. Mitochondria (2 mg/ml) suspended in the cross-linking medium were treated with freshly prepared (100 μl/ml) 1,5-difluro-2,4-dinitrobenzene at 25°C. 1,5-Difluoro-2,4-dinitrobenzene stock solution was made by dissolving 4.08 mg (20 mM) in 1 ml of the cross-linking medium (pH 7.4). 1,5-Difluro-2,4-dinitrobenzene was only partially soluble under these conditions. Reaction was quenched by the addition of ammonium acetate to a final concentration of 100 mM.

Cu^{2+}-o-phenanthroline. For cross-linking by oxidation of sulfhydryl groups by Cu^{2+}-*o*-phenanthroline, the mitochondria (2 mg/ml) were suspended in cross-linking buffer and aliquots from a stock solution of 13.6 mM *o*-phenanthroline and 6.8 mM cupric sulfate were added to a final concentration of 430 μM Cu^{2+}-*o*-phenanthroline in the reaction mixture. Cross-linking was quenched by the addition of EDTA solution to a final concentration of 10 mM. The free sulfhydryl groups were alkylated by the addition of freshly made *N*-ethylmaleimide (8 mM final concentration).

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide. Mitochondria (2 mg/ml) suspended in a medium containing 220 mM mannitol and 70 mM sucrose adjusted to pH 7.4 using KOH were treated with a fresh solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide to a final concentration of 30 mM. The cross-linking reaction was quenched by the addition of ammonium acetate solution at a final concentration of 200 mM.

All cross-linking reactions were carried out at room temperature. After quenching the reaction, the mitochondria were reisolated by centrifugation at 20000 × *g* for 10 min, and washed with the cross-linking buffer and finally, suspended in sodium phosphate buffer (0.1 M), pH 7.4.

Lysis of mitochondria

Native and cross-linked mitochondria were suspended in 0.5 ml of cold 0.1 M potassium phosphate buffer, pH 7.4, and sonicated for a total of 2 min (20-s sonication periods followed by periods of 15–30 s cooling of the tip as well as the materials) in a Sonicater cell disrupter model W-225R fitted with a microtip. Sonicated mitochondria were treated with 0.2% Triton and vigorously blended on a vortex mixer and placed in an ice bath for 10 min. The suspension was centrifuged at 30000 × *g* for 30 min and the supernatant fractions were saved as soluble nonsedimentable material. The precipitates were washed and resuspended in the above buffer.

Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out as described earlier [18] on an 8–13% exponential acrylamide gradient for the running gel and 4% acrylamide for stacking gel. For samples that contained proteins cross-linked through disulfide bridges, β-mercaptoethanol was excluded from the lysis buffer and *N*-ethylmaleimide (8 mM) was added prior to addition of SDS. The samples were then incubated at 37°C for 2 h before electrophoresis. Protein (30–40 μg) was used for electrophoresis. Protein samples were prepared for polyacrylamide gel electrophoresis immediately after cross-linking or were stored frozen at −20°C for subsequent electrophoresis.

For enzymatic staining, electrophoresis was carried out on native polyacrylamide gels (7.5%) according to the method of Davis [19]. Activity staining of malate dehydrogenase was carried out as described by Gabriel [20]. Fumarase was stained as described earlier [21,22]. The staining solution contained 0.4 M fumarate, 5 mM NAD, 0.3 mg/ml nitroblue tetrazolium, 0.013 mg/ml phenazine methosulfate, 0.05 mg/ml malate dehydrogenase in 0.1 M Tris-HCl, pH 7.5.

Enzyme assays

Citrate synthase [23], malate dehydrogenase [24] and fumarase [25] were assayed as described earlier. One unit of enzyme activity is defined as 1 μmol of product formed per min. Protein was determined by the method of Lowry et al. [26] using bovine serum albumin as the standard.

Results

Comparison of different cross-linkers

The ability of different cross-linkers, with varying arm lengths, specific for amino groups to

cross-link rat liver mitochondria was studied. These included methyl acetimidate as a control reagent, dissucinimidyl tartarate (6 Å), dimethyl adipimidate (9 Å), dimethyl pimelimidate (10 Å), dimethyl suberimidate (11 Å) and glutaraldehyde. The degree of cross-linking of mitochondrial proteins was ascertained by SDS-polyacrylamide gel electrophoresis (Fig. 1). The disappearance of several monomeric bands is evidence of the ability of the different cross-linkers to enter the mitochondria and cross-link matrix proteins. Especially noticeable is the dimunition of the band corresponding to 130000 molecular weight (CPS, Fig. 1) which is known to be carbamyl phosphate synthetase, the major liver mitochondrial matrix protein [27]. All the bifunctional cross-linkers used were found to cross-link mitochondrial proteins at 10 mM concentration at pH 7.4 even in a time interval as short as 2 min. The changes in the monomeric band patterns were almost identical with all the cross-linkers although dimethyl pimelimidate dihydrochloride, dimethyl suberimidate dihydrochloride, disuccinimidyl tartarate and glutaraldehyde caused a more extensive cross-linking, forming polymeric aggregates that failed to enter even the 4% stacking gel (T4 arrow, Fig. 1) and appeared as intensely staining bands at the top of the gels (also T8 arrow, Fig. 1). Methyl acetimidate hydrochloride, a monofunctional control reactant, did not, of course, cross-link mitochondrial proteins nor did it alter the gel pattern.

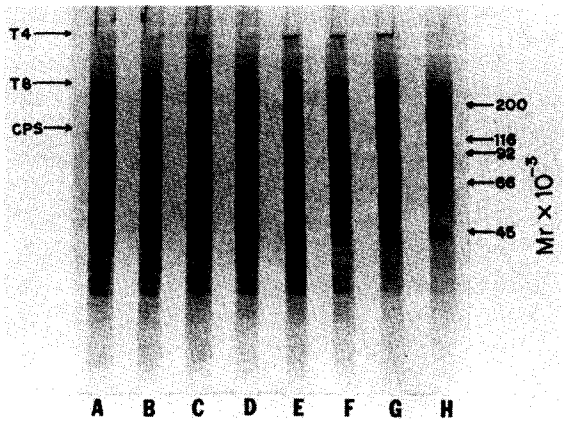

Fig. 1. SDS-polyacrylamide gel electrophoresis of rat liver mitochondria cross-linked with different cross-linkers. Cross-linking was carried out for 2 min with 10 mM concentration of different cross-linkers. (A) Native mitochondria, (B) methyl acetimidate hydrochloride, (C) disuccinimidyl tartarate, (D) dimethyl adipimidate dihydrochloride, (E) dimethyl pimelimidate dihydrochloride, (F) dimethyl suberimidate dihydrochloride, (G) glutaraldehyde, and (H) molecular weight markers: myosin, 200000; β-galactosidase, 116250; phosphorylase B, 92500; bovine serum albumin, 66200 and ovalbumin, 45000. T4 indicates the top of the 4% spacer gel; T8 indicates the top of the 8–13% running gel and CPS indicates the location of carbamoyl phosphate synthetase.

1,5-Difluoro-2,4-dinitrobenzene can form 3–5 Å bridges between amino groups and/or tyrosine phenolic groups [28,29]. 1,5-Difluoro-2,4-dinitrobenzene was found to cross-link some proteins as seen by the disappearance of band A in Fig. 2 (lane B compared to lane A) and appearance of a distinct higher molecular weight band B (lane B, Fig. 2). Cu^{2+}-*o*-phenanthroline catalyzes zero-length cross-links between proteins by facilitating the oxidation of free sulfhydryl groups which are in close proximity to form a disulfide bridge [1,29]. The close proximity of different mitochondrial

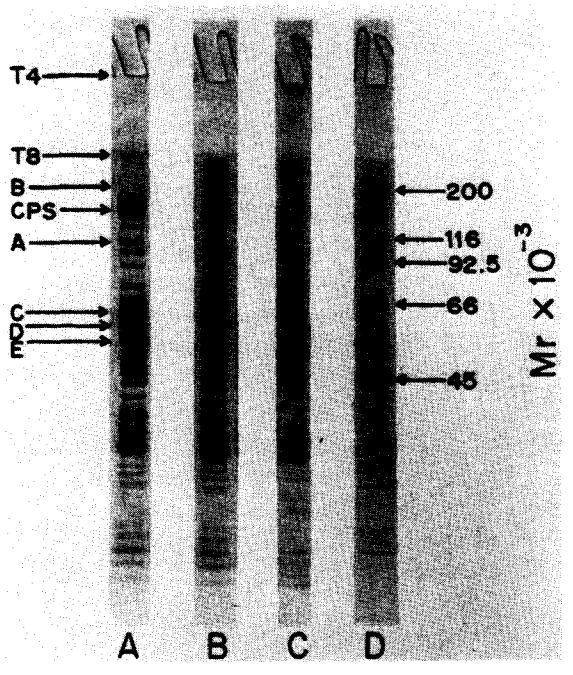

Fig. 2. SDS-polyacrylamide gel electrophoresis of cross-linked rat liver mitochondria. (A) Native mitochondria, (B) 1,5-difluoro-2,4-dinitrobenzene (2 min), (C) Cu^{2+}-*o*-phenanthroline (2 min), and (D) molecular weight markers as in Fig. 1. Experimental details are given in the text (see legend to Fig. 1 for explanation of T4, T8 and CPS). Bands designated A–E are explained in the text.

proteins is evident from the cross-linking pattern of Cu^{2+}-*o*-phenanthroline-oxidized mitochondria which indicates extensive cross-linking, both from the disappearance of monomeric bands (CPS, C and D) and appearance of protein bands at the top of the gel as nonpenetrable high molecular weight species (T4 and T8 arrows, lane C) (Fig. 2). Not all protein bands decrease (for example, lane C, band E, Fig. 2). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, a water-soluble carbodiimide, can form zero-length cross-links between proteins by promoting condensation bteween a free amino group and a free carboxyl group to form a peptide link [30]. Under the conditions employed, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride was not an effective cross-linking reagent. Since 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride promotes a condensation reaction by acid-catalyzed removal of water, its ability to cross-link mitochondrial proteins at pH 6 was studied but it was not effective even at pH 6. However, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride can cross-link mitochondrial proteins if cross-linking is carried out for more than 30 min using 30 mM concentration of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride. The results at these higher concentrations and times were found to be identical at pH 6 and 7.4 (results not shown).

Studies with glutaraldehyde

Among the different cross-linkers used, glutaraldehyde was found to be the most potent one, and its effect on rat liver mitochondria was evaluated more fully. Glutaraldehyde is known to react with free amino groups [1,31] and a decrease in about 40% of amino groups was observed when mitochondria were cross-linked for 5 min with 0.02% glutaraldehyde. Osmotic stability of mitochondria was achieved when 40% of the free amino groups were cross-linked by glutaraldehyde.

Lysine, glycine and Tris [32–34], which are commonly used as quenchers in long time interval studies with glutaraldehyde, were neither rapid nor efficient enough for use in the present studies. For these experiments where cross-linking was carried out for very short times, it was necessary to use hydrazine to achieve rapid quenching for the cross-linking reaction.

Cross-linking mitochondria in different conformations

Isolated mitochondria are known to undergo physiologically induced changes in size (volume). We determined whether such changes resulted in alteration in the cross-linking of matrix proteins. Mitochondria were incubated with ADP for 5 min and were cross-linked with glutaraldehyde (0.02%) for 2 min in the presence of ADP which is known to keep the mitochondria in a condensed state [35]. The SDS-polyacrylamide gel electrophoresis pattern (Fig. 3) indicates that ADP enhanced the amount of cross-linking under the conditions used as indicated by the increase in the amount of

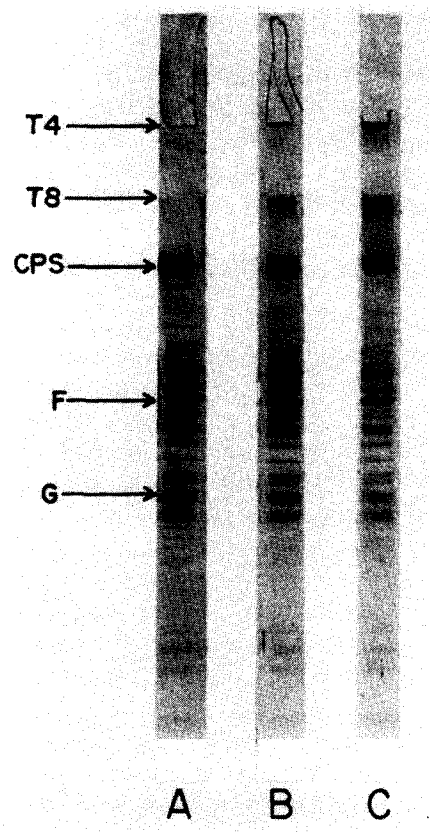

Fig. 3. SDS-polyacrylamide gel electrophoresis of rat liver mitochondria cross-linked in the presence of different metabolites. Mitochondria (2 mg/ml) were incubated for 5 min with different metabolites, after which they were cross-linked for 2 min with 0.02% glutaraldehyde. (A) Native mitochondria, (B) cross-linked mitochondria, (C) 1 mM ADP (see legend to Fig. 1 for explanation of T4, T8 and CPS – see text for band F).

protein not penetrating the gel (T4 and T8 arrows) and the decrease in the amount of the 130000 molecular weight protein band (CPS). It can be seen that most of the CPS band protein disappears whereas other monomers are relatively unreacted (band F, for example).

Earlier studies from this laboratory have indicated that in swollen mitochondria, gross disorganization of the matrix occurs [16]. In order to evaluate whether such changes result in changes in the cross-linking of mitochondrial proteins, mitochondria at different stages of swelling were cross-linked with 0.1% glutaraldehyde for 2 min after addition of 5 mM $MgCl_2$ (Fig. 4). The SDS-polyacrylamide gel electrophoresis pattern (Fig. 4) indicates that swelling of the mitochondria resulted in a decrease in the amount of cross-linking of their proteins with little impenetrable protein (T4 and T8 of lane C and D) and little decrease in CPS (lanes C and D). There was not much change in the amount of cross-linking pattern on further incubation from time C to time D (Fig. 4). Sonicated, cross-linked mitochondria indicated a drastic reduction in the rate of cross-linking (data not shown).

The electrophoretic patterns of the mitochondrial subfractions (2 mg/ml) cross-linked with 0.02% glutaraldehyde for 5 min are shown in Fig. 5. When the intact mitochondria (lane B), mitoplasts (lane D) and inner membrane (lane L) were cross-linked by glutaraldehyde, nonpenetrating protein (T4 and T8) was formed and a decrease in lower molecular weight components (CPS, for example) occurred. It is interesting to note that no extensive cross-linking of the matrix fraction (lane F) was seen under these conditions as evidenced by very little protein at T4 and T8 and no reduction in CPS. However, unlike the cross-linked intact mitochondria, matrix protein gave rise to a number of minor bands in the higher molecular weight region (lane F region above CPS and below T8).

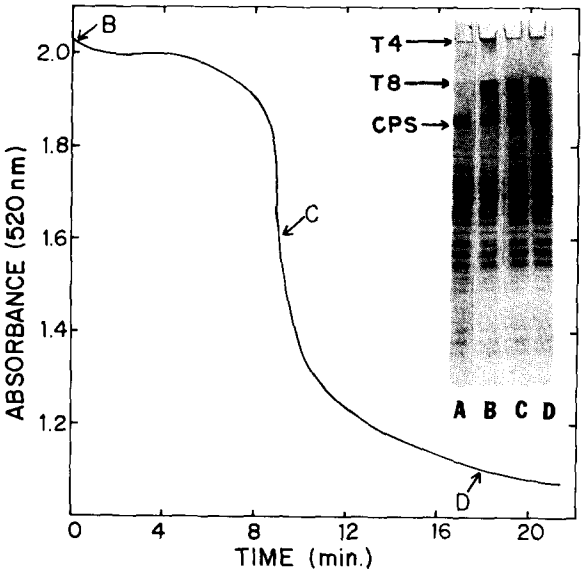

Fig. 4. SDS-polyacrylamide gel electrophoresis of swollen rat liver mitochondria cross-linked with glutaraldehyde. See text for the details on swelling. At different times of swelling (at the points indicated B–D) mitochondria were cross-linked for 2 min using 0.1% glutaraldehyde. (A) Native mitochondria (see legend to Fig. 1 for explanation of T4, T8 and CPS).

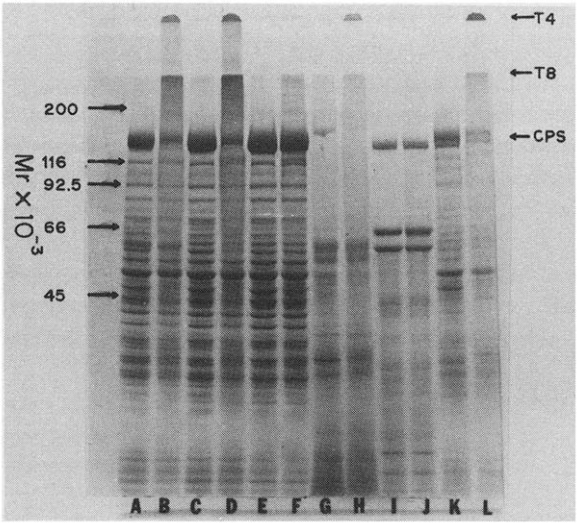

Fig. 5. SDS-polyacrylamide gel electrophoresis of cross-linked rat liver mitochondrial subfractions. Mitochondrial subfractions (1–2 mg/ml) were cross-linked for 5 min with 0.02% glutaraldehyde. Mobility of molecular weight markers (as in Fig. 1), (A) native mitochondria, (B) cross-linked mitochondria, (C) native mitoplast, (D) cross-linked mitoplast, (E) native matrix, (F) cross-linked matrix, (G) native outer membrane, (H) cross-linked outer membrane, (I) native intermembrane space proteins, (J) cross-linked intermembrane space proteins, (K) native inner membranes, (L) cross-linked inner membranes (see legend to Fig. 1 for explanation of T4, T8 and CPS).

Cross-linking of matrix proteins, citrate synthase, malate dehydrogenase and fumarase to sedimentable fractions in mitochondria

In order to ascertain whether the matrix proteins including Krebs cycle enzymes were cross-linked, the mitochondria (native or cross-linked) were lysed, and membrane and cross-linked material were sedimented at 30 000 × *g* for 30 min (see Materials and Methods). The supernatant fraction was saved as the nonsedimentable soluble matrix. Analysis of these two fractions indicated an increase in the protein in the membrane fraction with a concomitant decrease in the soluble matrix fraction (Fig. 6). About 75% of the total liver mitochondrial protein was found in the sedimented membrane fraction after cross-linking, thus indicating that cross-linking of matrix protein had occurred. Larger amounts of protein are cross-linked in heart mitochondria as compared to liver mitochondria (Fig. 6).

The activity of the Krebs cycle enzymes, citrate synthase, malate dehydrogenase and fumarase, was measured in both sedimentable and soluble fractions. The activity of all the three enzymes increased in the precipitates after corss-linking of liver mitochondria with a concomitant decrease in activities in the supernatant fractions (Fig. 7). Specific activities of the enzymes in the precipitate and supernatants of cross-linked rat liver and heart

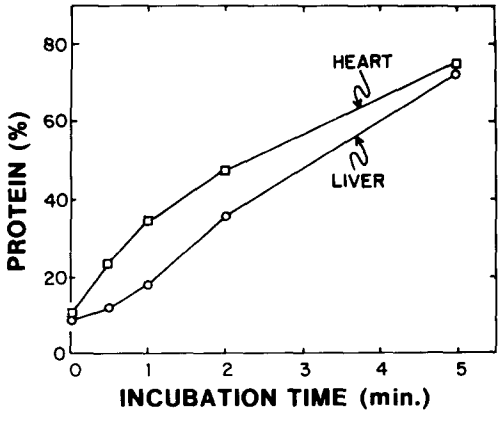

Fig. 6. Relative amounts of protein in the precipitates of rat liver and heart mitochondria cross-linked with glutaraldehyde. Rat liver or heart mitochondria (2 mg/ml) were cross-linked with glutaraldehyde, lysed and the precipitates and supernatant fractions were obtained as described in the text. Total protein in each fraction was taken as 100% for comparison.

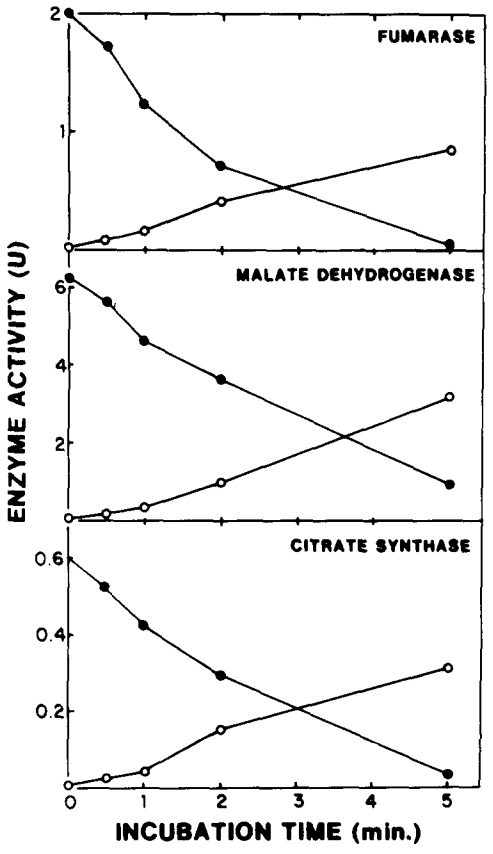

Fig. 7. Activities of some Krebs cycle enzymes in the supernatant fractions and precipitates of rat liver mitochondria cross-linked with glutaraldehyde. Rat liver mitochondria (3.4 mg) were cross-linked with glutaraldehyde (0.02%), lysed and precipitates and supernatant fractions were obtained as described in the text. (●———●) Supernatant, (○———○) precipitate.

mitochondria are shown in Table I. The cross-linking of heart mitochondria gave a precipitate fraction containing the three enzymes with the specific activities of citrate synthase and malate dehydrogenase lower than those in the supernatant fractions and fumarase activity about the same, while the specific activities of all three enzymes are higher in the precipitate than the supernatant fraction with cross-linked liver mitochondria.

Citrate synthase, malate dehydrogenase and fumarase are inactivated to some extent by glutaraldehyde treatment. The extent of inactivation was found to be greater when intact mitochondria were cross-linked with glutaralde-

TABLE I

SPECIFIC ACTIVITIES OF ENZYMES AFTER GLUTARALDEHYDE TREATMENT OF RAT MITOCHONDRIA

Mitochondria (2 mg/ml) were cross-linked for 5 min with 0.02% glutaraldehyde, lysed and precipitates and supernatant fractions were obtained as described in the text. Each value represents the mean ± S.E. from three experiments.

Enzyme	Specific activity (U/mg protein)			
	Heart		Liver	
	Supernatant	Precipitate	Supernatant	Precipitate
Citrate synthase	2.0 ±0.20	1.4 ±0.10	0.028 ± 0.005	0.12 ± 0.01
Malate dehydrogenase	5.5 ±0.35	2.1 ±0.15	0.91 ± 0.07	1.24 ± 0.08
Fumarase	0.66 ± 0.03	0.77 ± 0.04	0.02 ± 0.01	0.32 ± 0.02

hyde as compared to glutaraldehyde cross-linking of sonicated mitochondrial preparations (Table II). Although the inactivation of these three enzymes was comparable in sonicated mitochondrial preparations treated with glutaraldehyde, the extent of inactivation of fumarase as compared to citrate synthase and malate dehydrogenase was found to be greater when intact mitochondria were treated with glutaraldehyde.

Effect of cross-linking on enzyme release from toluene-treated mitochondria

Previous studies from this laboratory have indicated that mitochondria could be made permeable to normally nonpenetrating metabolites by treatment with toluene [16]. However, most of the matrix enzymes were found to leak out of toluene-treated mitochondria when they were suspended in isotonic buffer solutions. This leakage could be prevented by the addition of 8.5% poly(ethylene glycol) [16]. In order to test the effect of cross-linking on the leakage of mitochondrial Krebs cycle enzymes under these conditions, the following experiments were performed. Rat liver mitochondria were cross-linked with glutaraldehyde (0.02%) for 5 min as described in the text. The native as well as the cross-linked mitochondria were then treated with toluene. The enzyme activities were measured in the supernatant solutions obtained after toluene treatment. The data indicate that leakage of the enzymes during toluene treatment was less in cross-linked mitochondria than the native mitochondria (Table III). In order to study the extent of leakage of enzymes after toluene treatment, a concentrated suspension of the toluene-treated mitochondria (20

TABLE II

INACTIVATION OF SOME KREBS CYCLE ENZYMES ON GLUTARALDEHYDE CROSS-LINKING OF INTACT AND SONICATED MITOCHONDRIA

Mitochondria, intact or sonicated (2 mg/ml), were cross-linked with 0.02% glutaraldehyde as described in the text. Intact mitochondria were lysed before assaying the enzyme activity as described in the text. The values in brackets indicate the % original activity remaining after cross-linking. One unit of enzyme activity is defined as 1 μmol of product formed per min.

Cross-linking time (min)	Enzyme activity (U)					
	Citrate synthase		Malate dehydrogenase		Fumarase	
	Intact	Sonicated	Intact	Sonicated	Intact	Sonicated
0	0.32 (100)	0.32 (100)	3.29 (100)	3.49 (100)	0.96 (100)	1.03 (100)
0.5	0.31 (97)	0.32 (100)	3.19 (97)	3.49 (100)	0.74 (77)	0.99 (96)
1	0.26 (81)	0.3 (94)	2.8 (85)	3.21 (92)	0.54 (56)	0.90 (88)
2	0.23 (72)	0.27 (84)	2.69 (82)	3.11 (89)	0.44 (46)	0.82 (80)
5	0.17 (53)	0.25 (78)	1.98 (60)	2.76 (79)	0.32 (33)	0.69 (67)

TABLE III

RELEASE OF SOME KREBS CYCLE ENZYMES FROM NATIVE AND CROSS-LINKED MITOCHONDRIA ON TOLUENE TREATMENT

Enzyme	% activity released [a]			
	During toluene treatment [b]		After toluene treatment [c]	
	Native	Cross-linked [d]	Native	Cross-linked [d]
Citrate synthase	14	5	80	28
Malate dehydrogenase	18	7.6	88	21
Fumarase	18	0	100	0

[a] Percentages are based on the total enzyme activity obtained on sonication of the native and cross-linked mitochondria, respectively.
[b] Mitochondria were treated with toluene as described in the text, resedimented and the enzyme activity in supernatant estimated.
[c] Native and cross-linked mitochondria (2 mg/ml) were incubated at room temperature in toluene treatment medium for 15 min, centrifuged at 20000 × *g* for 5 min, and activities in the supernatants estimated.
[d] Mitochondria (2 mg/ml) were cross-linked with 0.02% glutaraldehyde for 5 min.

mg/ml) was diluted to 2 mg protein per ml in a medium containing 220 mM mannitol, 70 mM sucrose, 2 mM Hepes (pH 7.4), 50 mM Tris-HCl (pH 7.4), 1 mM EDTA and 0.05% bovine serum albumin and incubated at 0°C. After 15 min, the suspension was centrifuged at 10000 × *g* for 10 min and supernatant solutions were assayed for enzyme activities (Table III). The results show a decrease in the release of different enzyme activities after cross-linking of the mitochondria. Of the three enzymes studied, fumarase was found to be completely retained in the cross-linked mitochondria treated with toluene. Treatment of mitochondria with glutaraldehyde alone was not found to alter its permeability characteristics as judged by enzymatic activity studies. The matrix enzyme activities unmasked by toluene treatment of mitochondria were about the same as those of sonicated mitochondria under identical assay conditions. It does appear, therefore, that the matrix enzymes are cross-linked to form either very large complexes or are perhaps cross-linked to the inner membrane, thus preventing their leakage.

Analysis of the nonsedimentable soluble fraction

The soluble fractions obtained from cross-linked rat liver mitochondria were analyzed for nonsedimentable soluble protein complexes by the enzymatic staining technique on nondenaturing polyacrylamide gels (see Materials and Methods). These studies indicated the disappearance of monomeric fumarase activity band (FUM) and its

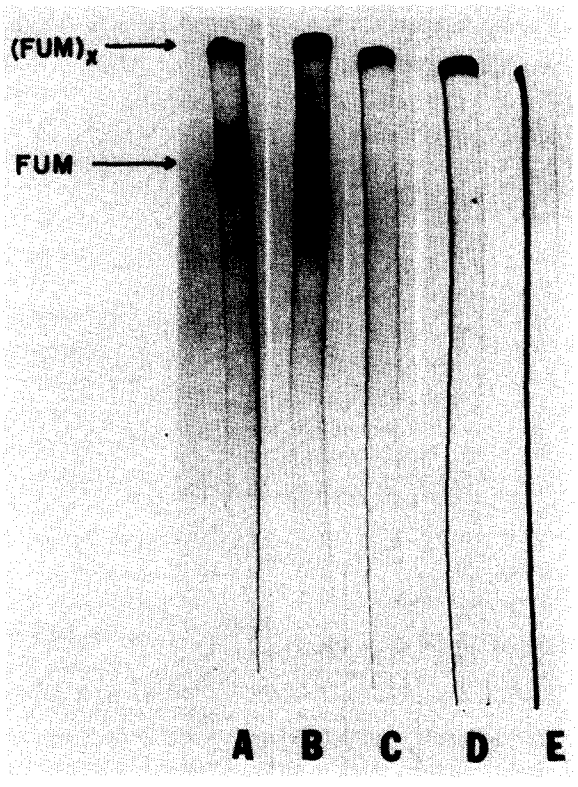

Fig. 8. Polyacrylamide gel electrophoresis of fumarase in native and cross-linked rat liver mitochondria. Rat liver mitochondria (2 mg/ml) were cross-linked with 0.02% glutaraldehyde, washed and resuspended in 0.5 ml of potassium phosphate buffer, pH 7.4, lysed and supernatant fractions were obtained as described in the text. Electrophoresis of the supernatant fractions and the fumarase activity staining were carried out as described in the text. 10 μl of the supernatant fractions were used for electrophoresis. (A) 0, (B) 0.5, (C) 1, (D) 2 and (E) 5 min.

appearance on the top of the gel as nonpenetrable material (FUM_x) (Fig. 8). After 2 min of cross-linking, even though there is a considerable amount of fumarase activity still present in the supernatants (Fig. 7), all of it seemed to have been cross-linked (unable to enter the gel, top arrow, Fig. 8) as seen from enzymatic staining data. Similar results were also obtained with gels stained for malate dehydrogenase enzyme activity. SDS-polyacrylamide gel electrophoresis of the soluble fraction indicated higher molecular weight polymers which failed to enter the gel, thus indicating that a part of the enzyme in the soluble fraction also contains cross-linked proteins with a large particle size (data not shown).

Discussion

The treatment of mitochondria and mitoplasts with a variety of cross-linking reagents results in the formation of high molecular weight protein complexes, some of which cannot enter a 4% SDS-polyacrylamide spacer gel and some of which cannot enter the 8% SDS-polyacrylamide gel. Concomitant with these changes, certain matrix proteins decrease in concentration (i.e., the carbamyl phosphate synthetase, M_r 130 000) while others seem to be relatively unchanged in their concentrations. Molecular weight complexes in the region between 130 000 and about 500 000 do not seem to form. Treatment of mitochondria that are swollen shows a decrease in the changes just noted while mitochondria with a condensed matrix shows an enhancement of these changes.

Cross-linking of matrix proteins in the absence of the inner membrane showed little formation of the large complexes (those not entering the 4% gel and those not entering the 8% gel) but showed an increase in what we term intermediate complexes (i.e., entering the 4% gel and 8% gel, but larger than M_r 130 000). Treatment of inner membranes showed the formation of both the 4 and 8% gel-impermeant components.

Additional information about the disposition of matrix proteins was obtained by following the activities of citrate synthase, malate dehydrogenase and fumarase after cross-linking of mitochondria. Several interesting results were obtained. Firstly, the enzymes were more susceptible to inactivation when the intact mitochondria were treated with glutaraldehyde than when the sonicated mitochondria were treated with glutaraldehyde. Secondly, cross-linking reduces the leakage of the enzymes from toluene treated mitochondria. Thirdly, treatment of heart mitochondria gave a precipitate fraction containing the three enzyme activities with specific activities lower than or equal to those of the supernatant fractions while the specific activities of these three enzymes are higher in the precipitate with cross-linked liver mitochondria.

One objection to this experimental approach is that even at cross-linking times as short as 2 min, random collisional protein interactions might result in a host of nonspecific cross-linkings. However, no concrete evidence has been produced for the occurrence of random collisional cross-linking in any system [36,37]. The absence of complexes above M_r 130 000 and below M_r 500 000 in cross-linked mitochondria and mitoplasts also argues against such random events.

Another question arises as to whether the impenetrable complexes are membrane protein complexes, membrane protein-matrix protein complexes, matrix protein complexes, or mixtures of these three. We have no direct evidence on this question, but the fact that cross-linking of matrix proteins alone does not give impenetrable protein material but rather gives intermediately large size complexes argues against the third possibility.

Another question is whether or not the changes seen when different physiological states of the mitochondria are cross-linked are due to changes in protein concentration within the matrix. We have no evidence at present to answer this question.

It should be remembered that cross-linking experiments with enzyme mixtures have shown a strong predilection toward intramolecular cross-linking versus intermolecular cross-linking [38]. The concentration of mitochondrial matrix proteins is very high and as a result, it is probable that there is little chance for rapid movement of these molecules in the matrix compartment. Recent experiments have demonstrated interactions between metabolically related sequential enzymes, e.g., citrate synthase and malate dehydrogenase [39]. We also have been able to demonstrate binding of

citrate synthase, malate dehydrogenase and fumarase to the inner surface of the mitochondrial inner membrane (D'Souza, S.F. and Srere, P.A., unpublished data). These observations coupled to the experiments here in which we show higher specific activities of the precipitable enzymes after cross-linking of liver mitochondria indicate to us that these enzymes have a preferential location in the matrix of being next to the inner membrane. Calculations based on stereomorphometric measurements indicate that in heart mitochondria, almost all the matrix protein is adjacent to the inner membrane whereas in liver mitochondria, the measurements indicate that less than 20% is next to the membrane [11]. These calculations explain the difference we have noted between the cross-linked heart and liver mitochondria regarding the specific activities of the precipitable Krebs cycle enzymes.

Enhanced inhibition of fumarase and its ability to be retained completely by the cross-linked mitochondria on toluene treatment as compared to citrate synthase and malate dehydrogenase suggests that fumarase is getting cross-linked more efficiently than the other enzymes. This might suggest closer proximity or association of fumarase with the inner membrane or closer proximity of glutaraldehyde-reactive groups. The earlier studies of Matlib and O'Brien [40] have indicated that fumarase activity is unmasked at low concentrations of digitonin as compared to the concentrations necessary for the unmasking of citrate synthase and malate dehydrogenase, thus indicating the proximity of fumarase to the inner membrane.

Thus, these cross-linking studies have indicated a preferential membrane location of Krebs cycle enzymes in the liver and close proximity of all proteins in the matrix. Further studies with cleavable cross-linkers and with photoactivatable affinity cross-linkers are being planned to obtain a more detailed localization of these enzymes.

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