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Fluorescent Labeling of Mitoplast Membrane. Effect of Oxidative Phosphorylation Uncouplers†

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ABSTRACT: Mitoplasts isolated from rat liver mitochondria were treated with fluorescamine at ratios ranging from 10 to 140 nmol per mg of protein. The labeled mitoplasts were separated into membrane and soluble fractions by osmotic lysis. The labels were found mainly in the membrane fraction. The soluble fraction had a negligible amount of the fluorescent labels even though the isolated soluble fraction could be labeled by fluorescamine to about half of the extent as could the membrane fraction. Thus, fluorescamine can only label exposed primary amino groups on the outside surface of mitoplasts. In the presence of succinate or β-hydroxybutyrate the extent of labeling of the membrane increases about 30%. This substrate-induced enhancement is prevented by oxidative phosphorylation uncouplers at uncoupling concentrations. Further analysis of labeled membrane showed that about 50% of the labels were associated with proteins and the remainder with lipids. This distribution remains unchanged as the labeling conditions vary, e.g., in the presence or absence of substrates or uncouplers. The protein labeling patterns showed that there are four major labeled polypeptides with apparent molecular weights of 49 000, 34 000, 24 000, and 14 000, respectively. These results suggest that energization of the mitoplast membrane exposes more positively charged primary amino groups on the outside surface and that this is achieved by rearranging the membrane proteins and lipids.

Fluorescamine, which reacts specifically with primary amines (Udenfriend, et al., 1972; Weigele et al., 1972), has been shown to label the surface of erythrocytes (Nakaya et al., 1975) and chick embryo fibroblasts (Hawkes et al., 1976). Studies on fluorescamine labeling of protein in $NaDodSO_4$[1] complexes suggest that a highly charged membrane surface could prevent the reagent from passing through the biomembranes (Tu & Grosso, 1976). Fluorescamine reacts nearly quantitatively with primary amines in aqueous media (the "labeling efficiency", defined by Weigele et al. (1972), is 85–90%). Thus, it can be used to titrate exposed primary amines.

Mitochondrial inner membrane can be separated by digitonin fractionation as described by Schnaitman & Greenawalt (1968), Chan et al. (1970), and Greenawalt (1974). The isolated inner membrane fraction or mitoplasts contain matrix protein enclosed in the membrane vesicles. Those authors also showed that mitoplasts can undergo respiration-dependent configurational changes, including changes from orthodox (expanded) to condensed (contracted) configurations, concomitant with respiratory transitions from state 4 to state 3. Uncouplers of oxidative phosphorylation can also induce changes of mitoplasts from orthodox to condensed configurations (Greenawalt, 1974) like those seen in intact mitochondria (Hackenbrock, 1966; Harris et al., 1968; Blair & Munn, 1972). The configurational changes induced by the different energy states could be associated with organizational changes of either the membrane proteins, as emphasized by Weinbach & Garbus (1969), Wilson & Azzi (1968), Wilson (1969), Wilson & Brooks (1970), and Hanstein & Hatefi (1974), or the lipids, as suggested by Chen & Hsia (1974), Bakker et al. (1975), Terada (1975), and Zimmer et al. (1972). Using lipid and protein spin labels, Zimmer (1977) observed that both protein and the lipid participate in structural changes induced in the mitochondrial membrane by FCCP.

In the present report, the structural changes of mitoplast membranes under different metabolic conditions were exam-

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[1] Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; S-13, 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; fluorescamine, 4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione; MAO, monoamine oxidase.

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ined by the use of a fluorescamine labeling technique. The results show that (1) more primary amine groups are titrable in the presence of succinate or β-hydroxybutyrate and (2) uncouplers decrease the labeling extent of both protein and lipid fractions.

Materials and Methods

Preparation of Mitoplasts. Rat liver mitochondria were prepared from male rats weighing 200–250 g according to the procedure of Weinbach (1961) and Johnson & Lardy (1967) as modified by Cross (1970). Mitoplasts essentially free from outer membrane contamination were prepared from rat liver mitochondria using digitonin by the method of Greenawalt (1974). The mitoplasts were then twice washed and resuspended in isolation medium which contained 220 mM mannitol, 70 mM sucrose, and 2 mM Hepes, at pH 7.4. The monoamine oxidase (MAO) activity of the mitoplasts and outer membrane fraction was assayed by the method of Tabor et al. (1954). The production of benzaldehyde from benzylamine in 50 mM sodium phosphate (pH 7.6) was monitored spectrophotometrically at 250 nm using an American Instruments DW-2 spectrometer at 25 °C. Protein concentrations were determined by the biuret method.

Fluorescent Labeling of Mitoplasts. To 3 mL of mitoplasts, suspended (~10 mg protein) in isolation medium with or without the presence of substrates or uncouplers, small amounts of fluorescamine in acetone solution were added according to the labeling procedure described by Tu & Grosso (1976). The membrane fraction and soluble proteins were separated, where necessary, by lysis of the mitoplasts (dilution with 10 volumes of 2 mM Hepes, pH 7.4, and incubation for 20 min at 0 °C) and centrifugation at 144 000*g* at 0 °C for 1.5 h. The fluorescence of each fraction was determined in 2 mM Hepes solution, pH 7.4, containing 0.1% $NaDodSO_4$. Since, as will be seen, internal labeling was negligible, the effect of energy state on labeling was determined without lysis.

Lipid Extraction of Labeled Mitoplast Membrane. Labeled mitoplast membrane isolated by the procedure mentioned above was further fractionated into lipid and protein parts according to the method of Lenaz et al. (1972). The membrane of labeled mitoplasts was treated with 10 mL of 5 M propanol at 0 °C for 10 min. Then, the lipid fraction was separated from proteins by centrifuging at 144 000*g* for 30 min using a Beckman 42-1 rotor. The distributions of fluorescent labels in these fractions were determined from fluorescence intensity measurements.

Quenching Correction of Fluorescence Measurements. Fluorescamine labeled glycine was synthesized by mixing an acetone solution of fluorescamine and an aqueous solution of glycine containing triethylamine according to the method of Weigele et al. (1972). The relative quantum yields of this standard fluorescent compound in different media were determined and were used to correct for the quenching of the mitoplast fluorescent labels. The excitation wavelength was fixed at 390 nm and the integrated emission spectra were used to measure fluorescence intensities. A Perkin-Elmer MPF-44A spectrofluorometer equipped with a differential corrected unit was used. In some earlier work, a Farrand Mark I spectrofluorometer with correction unit was also used.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Labeled mitoplast membrane and soluble fractions separated by osmotic lysis procedure were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis according to the procedure of Weber & Osborn (1969). Proteins dissolved in 0.1 M phosphate, pH 8.0, containing 1% $NaDodSO_4$ and 0.2% mercaptoethanol, were incubated at 37 °C for 12 h before being applied to a 7.5% polyacrylamide gel containing 0.2% cross-linker. For the approximate molecular weight determination of separated subunits, dansylated bovine serum albumin (mol wt 68 000), ovalbumin (mol wt 43 000), carbonic anhydrase (mol wt 29 000), and lysozyme (mol wt 13 930) were used as internal calibration markers. After electrophoresis, some gels were stained with Coomassie Brilliant Blue and later destained in a Bio-Rad diffusion destainer. The stained polypeptide bands were located by scanning at 550 nm with an ISCO Model 659 gel scanner attached to an ISCO Model UA-5 monitor. The fluorescence patterns of the protein fraction of labeled mitoplast membrane were determined by using a modified ISCO Model 659 gel scanner. The emission intensity was recorded with a Varian A-25 recorder which was connected to an RCA 1P-21 photomultiplier. A cut-off filter (<425 nm) was placed in front of the photomultiplier to minimize the scattering of incident light.

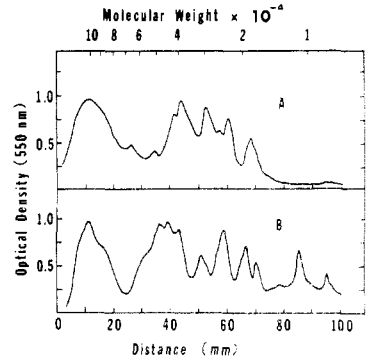

FIGURE 1: Gel electrophoresis of mitoplast membrane and soluble fraction. Mitoplast membrane and soluble fraction were prepared according to the procedures described in Materials and Methods. After gel electrophoresis, the gels were stained and destained and then the gel patterns were measured by an ISCO gel scanner. (A) Pattern of soluble fraction, and (B) pattern of membrane fraction. For molecular weight estimation, dansylated bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme were used.

Materials. Fluorescamine, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Pierce Chemical Co. Bovine serum albumin, ovalbumin, carbonic anhydrase, lysozyme, glycine, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) were obtained from Sigma Chemical Co. Electrophoresis grade sodium dodecyl sulfate, acrylamide, and *N,N'*-methylenebis(acrylamide) were purchased from Bio-Rad Laboratories. The benzylamine used for MAO activity analysis was freshly distilled. 5-Chloro-3-*tert*-butyl-2'-chloro-4'-nitrosalicylanilide (S13) was a generous gift of Dr. D. Durst of Bioenergetics Laboratory, SUNY at Buffalo.

Results and Discussion

Fractionation of Mitoplasts. Mitoplasts prepared according to the method of Greenawalt (1974) were washed twice with the isolation medium, leaving them essentially free of outer membrane contamination (with less than 1% of total MAO activity). In many experiments, mitoplasts were treated with fluorescamine at ratios varying from 0 to 100 nmol per mg of mitoplast protein. The amount of protein released into the soluble fraction (matrix proteins) by the lysis procedure mentioned was consistently 31 ± 4% of the total (matrix plus soluble), independent of fluorescamine concentration. The electrophoresis patterns of the membrane and the soluble fraction obtained by the present method are shown in Figure 1. Both fractions contain many different polypeptides and the soluble fraction has no polypeptide with apparent molecular

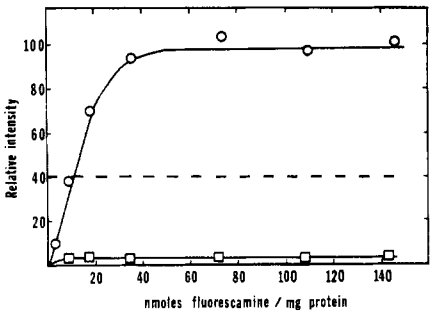

FIGURE 2: Extents of labeling of mitoplast membrane and soluble fraction. Mitoplasts were treated by fluorescamine at the indicated concentrations. The membrane and soluble fractions were separated by osmotic lysis method as mentioned in Materials and Methods. The extent of labeling is expressed in terms of the observed fluorescence intensity per mg of protein of each fraction. (O—O) Represents membrane fraction; (□—□) represents soluble fraction. The saturation level of the labeling of the separated soluble fraction is shown as dashed line.

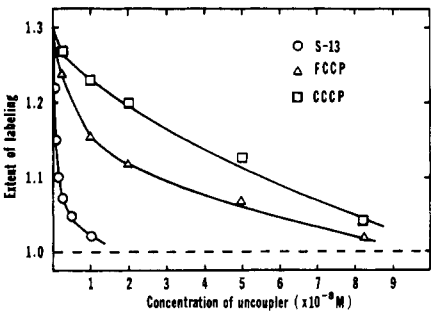

FIGURE 3: Effect of uncouplers on fluorescamine labeling. Mitoplasts were treated with 60 nmol of fluorescamine per mg of protein in the presence of 5 mM succinate or β-hydroxybutyrate and uncouplers with indicated concentrations at labeling stages. The extent of labeling fluorescence intensity per mg of protein of mitoplast membrane was determined as described in Materials and Methods. The level of labeling was compared with that obtained in the absence of substrates and uncouplers. This reference level is set as 1.0 and is indicated as a dashed line.

weight lower than 16 000.

Caplan & Greenawalt (1966) and Vashington & Greenawalt (1968) have reported that about 50% of the total rat liver mitochondrial protein can be removed by repeated water treatment. This result suggests that the total amount of soluble protein (matrix plus cytoplasmic) is about equal to the amount of protein in mitochondrial ghosts (inner and outer membranes). Slightly different results were obtained by Sottocasa et al. (1967). They reported that the total soluble fraction contains 33%, while the inner and outer membranes contain 58% and 9% of the total protein, respectively. These results indicate that the inner membrane has 40 to 60% of the total protein, or, from our results, that matrix protein is 30% of total mitoplast protein (matrix plus inner membrane); thus, the released matrix proteins represent 17 to 24% of total mitochondrial protein.

Labeling of Mitoplasts. Mitoplasts treated with fluorescamine were further fractionated as mentioned before. To test the permeability of fluorescamine, the fluorescence intensities of both soluble and membrane fractions were measured. The extent of labeling of these fractions is shown in Figure 2. It is clear that membrane fraction carried over 95% of the labeling. It is estimated from the labeling efficiency that there are about 32 to 36 nmol of titrable primary amines per mg of protein associated with the membrane part. The low labeling of the soluble fraction could be due either to the low permeability of the labeling reagent under our experimental conditions or to the low reactivity of the soluble proteins toward fluorescamine. When the separated soluble fraction was treated with excess fluorescamine, the extent of labeling reached about half the level of the membrane, as shown in Figure 2. Thus, our results indicate that the failure to label soluble proteins enclosed in mitoplast vesicles is not because of a low reactivity (lacking of titrable primary amines) toward fluorescamine but rather because fluorescamine cannot pass through mitoplast membrane. Similar labeling results were also observed in the presence of succinate or β-hydroxybutyrate.

Effects of Uncouplers on Labeling. The labeled groups must mainly reside on the exterior surface of the mitoplasts since fluorescamine cannot penetrate the membrane. It has also been shown that the labeling efficiency of fluorescamine toward primary amino groups is about 85 to 90% (Weigele et al., 1972; Tu & Grosso, 1976) in the aqueous media. Thus, exposed primary amino groups of mitoplast membrane can be titrated by fluorescamine.

Greenawalt (1974) reported that mitoplast membrane, like the inner membrane in intact mitochondria, has the ability to undergo respiration-dependent configurational changes including changes from orthodox (expanded) to condensed (contracted) configurations, concomitant with respiratory transitions from state 4 to state 3. These changes of the membrane surface in terms of the number of surface exposed primary amino groups were determined by fluorescamine labeling.

In the presence of succinate or β-hydroxybutyrate, the saturation level of mitoplast membrane labeling increased 30%, or 10 to 12 additional nmol of primary amine groups per mg of mitoplast protein (Figure 3). When uncouplers were also added, the extent of labeling decreased. At optimal concentrations for uncoupling of oxidative phosphorylation, the extent of labeling returned to the level obtained in the absence of both substrates and uncouplers. The most powerful uncoupler, S-13, is also the most efficient reagent to reverse the increased exposure of primary amine groups induced by the energization process. Thus, these observations suggest that the change of the number of exposed primary amine groups on the exterior surface of mitoplast membrane is closely related to the configurational changes induced by the energy states.

Origin of Labeling. The configurational changes observed under different energy states could be the results of either conformational changes in the membrane proteins as suggested by Weinbach & Garbus (1969), Wilson & Azzi (1968), Wilson (1969), Wilson & Brooks (1970), Hanstein & Hatefi (1974), or the loosening of the tightly packed phospholipid head groups as suggested by Zimmer et al. (1972) and Zimmer (1973) or both. A recent study of Zimmer (1977) on the structural changes of mitochondrial inner membrane induced by uncouplers, using lipid and protein spin labeling, indicated that both protein and lipid components are involved. This suggestion was tested by examining the distribution of the fluorescamine labeled groups in the mitoplast membrane.

The exposed primary amine groups of mitoplast membrane may include the N-terminal amino acids and ε-amino groups of lysine residues of the membrane proteins and the amine groups of phosphatidylethanolamine and phosphatidylserine of membrane lipids. The phospholipids of labeled mitoplast membrane can be extracted by propanol using the method of Lenaz et al. (1972), and the distribution of labeling between protein and lipid fractions can be determined from corrected fluorescence intensity measurements. The membrane fractions of labeled mitoplasts which were treated with 40 nmol of fluorescamine per mg of protein in the presence of (1) no substrates and uncouplers, (2) 10 mM succinate, and (3) 10 mM succinate plus 8.2×10^{-8} M CCCP were isolated and

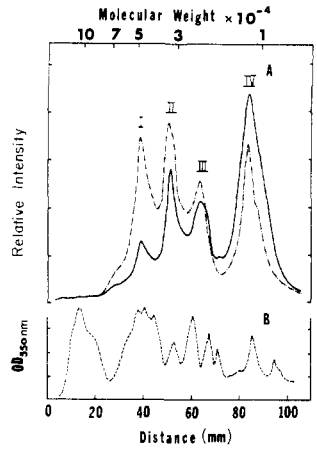

FIGURE 4: Labeling pattern of the protein fraction of mitoplast membrane. Mitoplast membrane was isolated from labeled mitoplasts and then treated with 1-propanol to remove the lipids. After gel electrophoresis, the labeling pattern of the protein fraction was recorded with a fluorescence scanner. (A) Pattern of labeled polypeptides: (—) mitoplasts treated with 80 nmol of fluorescamine per mg of protein in the presence of 5 mM succinate and 8.0×10^{-8} M of CCCP; (- - -) mitoplasts treated with 80 nmol of fluorescamine per mg of protein in the presence of 5 mM succinate. Same amount of total protein was applied to each gel. The apparent molecular weight of labeled polypeptide: (I) 49 000; (II) 34 000; (III) 24 000; and (IV) 14 000. Membrane proteins isolated from mitoplasts treated with the same amount of fluorescamine in the absence of succinate and CCCP were the same as in —. (B) Pattern of total mitoplast membrane proteins determined from Coomassie Brilliant Blue stained gel.

extracted with aqueous 1-propanol. The results showed that independent of the labeling conditions, there was 51 ± 2% of total fluorescence in the lipid fractions. Thus, both lipid and protein fractions have about the same amount of the labeling.

Since, as mentioned before, more fluorescamine-titrable amino groups are exposed in the presence of substrates, the above results indicate that the protein and lipid fractions contribute to the observed increase to about the same extent. Also, uncouplers can decrease the extent of labeling of both lipid and protein fractions with the same efficiency. Thus, the results are in agreement with Zimmer (1977) that both protein and lipid moieties participate in the configurational changes of mitoplast membrane and that both moieties may contribute to the uncoupling events.

After removing the lipid fraction from the labeled membranes, the protein residues were subjected to $NaDodSO_4$ gel electrophoresis. The fluorescence labeling patterns are shown in Figure 4. There are four main labeled polypeptides with the apparent molecular weight as 49 000 (I), 34 000 (II), 24 000 (III), and 14 000 (IV), respectively. Although the labeling of the protein fraction was enhanced by energization, the changes in individual labeled peptides were not uniform. We observed that peak I was strongly enhanced by energization, peaks II and III increased moderately, and peak IV actually decreased upon energization. The observation that both lipids and proteins participate in the same proportion in the changes tends to suggest that gross rearrangements of membrane may be responsible for the effect; e.g., more membrane becomes internal after the uncouplers are added. The present results suggest that perhaps the tighter packing of phospholipids and the inward motion of the surface proteins with higher molecular weight are partially responsible for the observed orthodox to condensed configurational changes in mitochondrial inner membrane.

In conclusion, the present report has demonstrated that fluorescamine can be used as a surface labeling reagent for mitoplast membrane. The fluorescamine titration data show that there are more primary amine groups exposed on the exterior surfaces of energized mitoplasts. The addition of oxidative phosphorylation uncouplers causes the rearrangement of energized membrane and decreases the amount of exposed primary amine groups. The analysis of the labeling extent of both protein and lipid fractions indicates that both fractions are involved in the uncoupler-induced configurational changes of mitoplasts. It is conceivable that a further analysis of labeled membrane components may provide detailed information about intramembranous motions of these molecules in the uncoupling event. This is currently being investigated.

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