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EFFECT OF CROSSLINKING ON MITOCHONDRIAL STRUCTURE AND FUNCTION

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Rat liver mitochondria were treated with ethylacetimidate* and methylbutyrimidate, monofunctional imidates, and with dimethylsuberimidate, a bifunctional imidate, and the effects on structure and function studied. Mitochondria treated with 5 mM dimethylsuberimidate or greater did not respond osmotically when placed in deionized water. Sodium dodecylsulfate-polyacrylamide gel electrophoresis revealed that at concentrations > 5 mM dimethylsuberimidate nearly all mitochondrial polypeptides failed to enter 6% gels, indicating crosslinking of both membrane and soluble proteins. Extensive amidination by ethylacetimidate and methylbutyrimidate had little effect on ascorbate-tetramethylphenylenediamine oxidase while extensive inhibition resulted from dimethylsuberimidate treatment. The possible involvement of molecular motion in electron transport is discussed.

INTRODUCTION

Earlier studies in our laboratory established that the lipid and protein components of the energy-transducing membranes of mitochondria and chloroplasts are mobile (1, 2). These studies, which employed a combination of spin labeling and freeze-fracture electron microscope techniques, correlated structural changes with changes in function. As a result, we have become more interested in the relationship between membrane mobility parameters and function.

One approach to investigate the importance of molecular motion in membrane function is to impose restrictions on motion by artificial means. One way this can be accomplished is by treating the systems with bifunctional alkylating agents. It is important, however, that these reagents do not affect enzymatic activity directly by alkylation. Recently a new class of alkylating agents have been described which appear to react with proteins in a mild and directed manner (3). These reagents, imidates, react primarily with free amino groups to yield cationic amidines, thereby conserving the original charge. Amidinated proteins exhibit minimal alteration of enzyme activity (4), antigenicity (5),

*Abbreviations: SDS, sodium dodecylsulfate; DMS, dimethylsuberimidate; EA, ethylacetimidate; MBI, methylbutyrimidate; TMPD, N, N, N′, N′-tetramethyl-p-phenylenediamine.

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and physical properties (6). Bifunctional imidates have been used to study the quaternary structure of oligomeric proteins (7), and the lateral association of erythrocyte membrane proteins (8, 9).

The present paper describes the effect of both monofunctional and bifunctional imidates on the structure and function of rat liver mitochondria.

METHODS

Materials

Dimethylsuberimidate dihydrochloride (DMS) and methylbutyrimidate hydrochloride (MBI) were purchased from Pierce Chemicals, Rockford, Ill. Ethyl acetimidate (EA) was generously provided by Peter Nemes, George Miljanich, and Dr. Edward Dratz, Department of Biochemistry, University of California, Santa Cruz. Fluram (fluorescamine) was obtained from Hoffmann-LaRoche, Nutley, N.J.

Isolation of Mitochondria

Mitochondria were isolated from rat liver using 0.33 M sucrose-1 mM EDTA (pH 7.5) as the isolation medium, as previously described (10). Mitochondria were washed once with 0.25 M sucrose prior to reaction with the imidoesters.

Imidate Treatment

Imidate hydrochlorides were dissolved in 0.133 M triethanolamine-HCl-0.33 M sucrose (pH 9.0) and the pH was adjusted immediately to 8.45–8.55 with NaOH. The stock solution was diluted with deionized water to give a final solution containing 0.10 M triethanolamine, 0.25 M sucrose, and 2–50 mM imidates. Solutions were used immediately.

Amidination was carried out by incubating mitochondria (2.5 mg protein/ml) with the imidates in 0.05 M triethanolamine-HCl-0.25 M sucrose (pH 8.5) for 30 min at room temperature (20–22°C). Following treatment, the reaction mixture was diluted 5-10 fold with ice cold 0.25 M sucrose and the preparations were reisolated by centrifugation (8,250 rpm, 10 min, 4°C, Sorvall SS34 rotor). The mitochondria were washed once in 0.25 M sucrose and suspended in this medium to 5–10 mg protein/ml.

Enzyme Assays

All activities were measured at room temperature in a reaction mixture containing 0.05 M Tris-HCl buffer (pH 8.0) and 0.25 M sucrose. Ascorbate-N, N, N′, N′-tetramethyl-p-phenylenediamine (TMPD) oxidase activity was assayed polarographically with 4 mM ascorbate and 0.3 mM TMPD.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed in gels containing 6% acrylamide and 0.1% sodium dodecyl sulfate (SDS) as previously described (11). Gels containing 4% acrylamide were prepared in a similar manner. Samples (1–2 mg protein/ml) were solubilized in 1% SDS and 1% mercaptoethanol. Gels were stained for protein with Coomassie brilliant blue (12).

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Osmotic Sensitivity

To examine the response of mitochondria to osmotic stress, samples (0.5–1.0 mg protein/ml) were incubated in either 0.25 M sucrose or deionized water. After equilibration for 15 min at room temperature, the optical density at 546 nm was recorded. The ratio (OD 546 [H_2O]/OD 546 [sucrose] was used as an empirical index of osmotic sensitivity.

Miscellaneous Analyses

Free amino groups were determined fluorometrically using the Fluram reagent by a modification of the method of Bohlen et al. (13). To assure that no amino group is sterically unavailable to the fluram reagent, SDS (1%) was added to the samples prior to the addition of the reagent. Protein was determined by the method of Lowry et al. (14) using bovine serum albumin as standard. In highly crosslinked material, SDS (1–2%) was added to the sample prior to the protein assay. The presence of SDS did not interfere with the assay.

RESULTS

Imidates react with primary amines to yield amidine linkages (Fig. 1). However, these reagents are highly unstable in aqueous solutions undergoing hydrolysis to a number of compounds. The instability of these reagents undoubtedly contributed significantly to the variation in the reactivity of the imidates from experiment to experiment. We, therefore, have related all imidate effects to the extent of amidination rather than the absolute concentration used.

Amidination of Mitochondria

Incubation of mitochondria with increasing concentrations of imidates resulted in the progressive loss of primary amino groups (Fig. 2). At comparable reactive concentrations, amidination by EA was greater than that obtained with DMS. Amidination by EA was complex. Incubation with 20 mM EA resulted in the loss of greater than 40% of mitochondrial amino groups with only an additional 25% amidination obtained by increasing the imidate concentration to 100 mM. Although not as apparent, a similar concentration dependence was observed with DMS. Under the conditions employed, MBI did not react as rapidly as either EA or DMS, with only 27% amidination noted at 100 mM MBI. The extent of reagent decay was not determined. However, it has been shown that methylacetimidate and DMS undergo hydrolysis with half-lives of 27 min (pH 8.0, 25°C, water) (3) and 42 min (pH 7.0, 25°C, 0.1 M triethanolamine) (15), respectively. These decay rates indicate that the bulk of the reagent was reactive throughout the incubation.

The reaction of DMS with mitochondria is complex and involves either bifunctional alkylation or monofunctional attack and hydrolysis. Since chemical analysis of amino group disappearance cannot distinguish between these two possible modes of interaction, SDS-polyacrylamide gel electrophoresis was employed to ascertain the degree of crosslinking of mitochondrial protein. Crosslinkage would result in the disappearance of monomer band and the appearance of new bands representing crosslinked polymers. Treatment with low concentrations of DMS (1 mM) resulted in the disappearance of

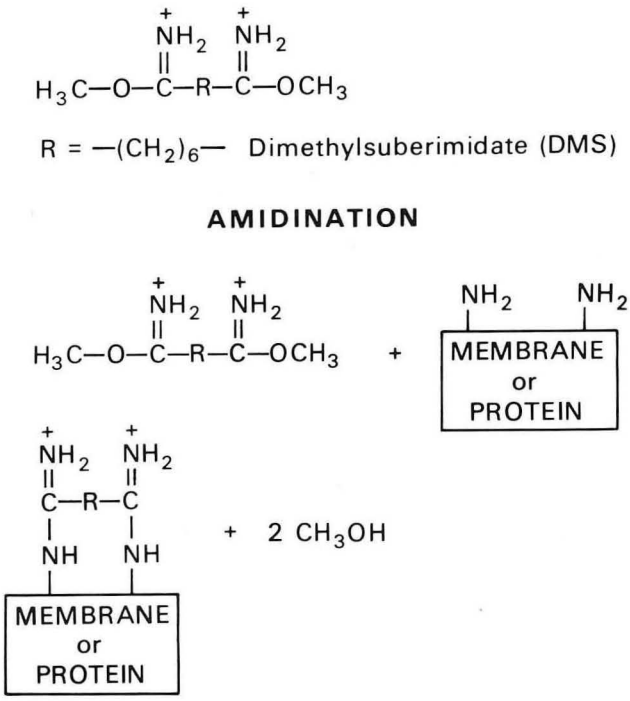

Fig. 1. Amidination reaction.

several bands corresponding to apparent molecular weights of 130,000, 60,000, 40,000 (Fig. 3). Extensive crosslinking of polypeptides occurred at concentrations of DMS greater than 5 mM. Since the 130,000 mol wt polypeptide crosslinked by DMS has been shown to be a major (30%) component of the mitochondrial matrix (11), these results indicate that components both of the inner membrane and the matrix are crosslinked by DMS. A considerable amount of material also failed to enter 4% acrylamide gels (mol wt exclusion ~ 500,000), demonstrating the high molecular weight of the crosslinked polypeptides. Treatment with EA at high concentrations caused some aggregation of mitochondrial polypeptides. This aggregation, which has been observed in other systems (4), can be reduced by solubilization of the samples immediately after preparation. The crosslinking of matrix proteins indicates that the imidates are permeable to the mitochondrial membrane systems.

Osmotic Properties of Amidinated Mitochondria

Mitochondria treated with higher concentrations of DMS were more difficult to resuspend than controls or EA-treated samples. Furthermore, DMS-treated preparations did not swell when placed in deionized water (Fig. 4). Osmotic insensitivity was obtained at 2.0–5.0 mM DMS. Monofunctional amidination was without effect on swelling. In some experiments, high concentrations of EA (100 mM) caused an apparent increase in mitochondrial osmotic fragility.

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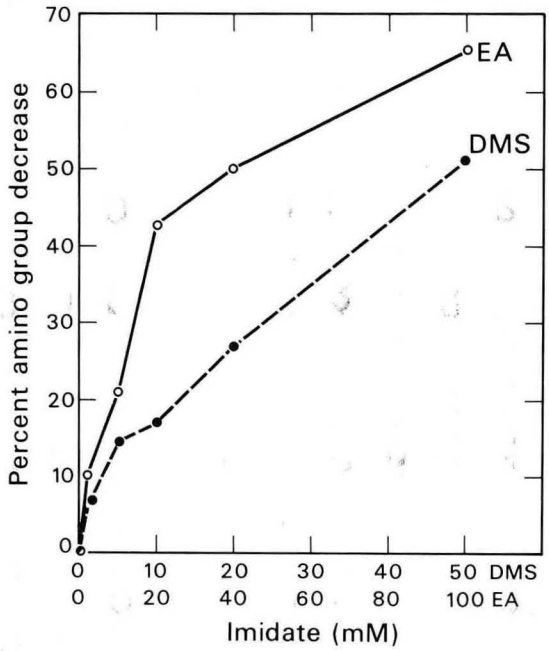

Fig. 2. Amidination of mitochondria. Mitochondria (2.5 mg protein/ml) were incubated with either EA or DMS in 0.05 M triethanolamine-HCl-0.25 M sucrose (pH 8.5) for 30 min at room temperature. Following incubation the samples were diluted with ice-cold 0.25 M sucrose and the mitochondria reisolated by centrifugation as described in Methods. Aliquots of the modified mitochondria were removed for protein and free amino group analysis. Amino group content is given as percent of untreated control. ●- - - -●, DMS; ○——○, EA.

Electron Transport Studies

Electron transport was measured to ascertain the functional integrity of amidinated mitochondria. Ascorbate-TMPD oxidase, which involves transfer of electrons from cytochrome c to oxygen, was selected for these studies because substrates need not be transported across the inner membrane prior to oxidation in this system. Effects induced by amidination can therefore be ascribed to direct modification of the electron transport system and not to a permease. Ascorbate-TMPD oxidase was relatively unaffected by treatment with either EA or MBI (Fig. 5). Amidination of 65% of mitochondrial amino groups by EA resulted in the loss of only 10% of oxidase activity while no effect was obtained with MBI (27% amidination). In contrast, treatment with increasing concentrations of DMS resulted in progressive loss of oxidase activity. Nearly 60% inhibition was noted at low levels of amidination (17%), with almost complete inhibition occurring when approximately 50% of mitochondrial amino groups were lost. It is interesting to note that at low concentrations of DMS (1–5 mM) considerable oxidase activity remained even though treatment resulted in loss of osmotic sensitivity and extensive crosslinking of mitochondrial protein. When different mitochondrial preparations were compared, some

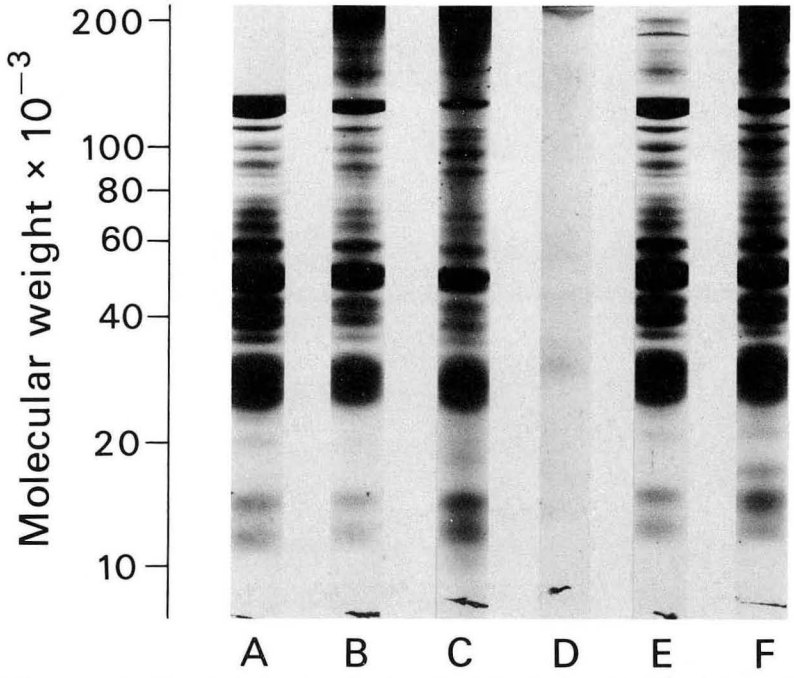

Fig. 3. SDS-polyacrylamide gel electrophoresis of amidinated mitochondria. Mitochondria (2.0 mg protein/ml) were solubilized in 1% SDS and 1% mercaptoethanol and subjected to electrophoresis in 6% polyacrylamide gels as described in Methods. Each gel contained 30 μg protein. Gels were stained with Coomassie brilliant blue. A. Control. B. 1 mM DMS. C, 5 mM DMS. D. 50 mM DMS. E. 2 mM EA. F. 100 mM EA.

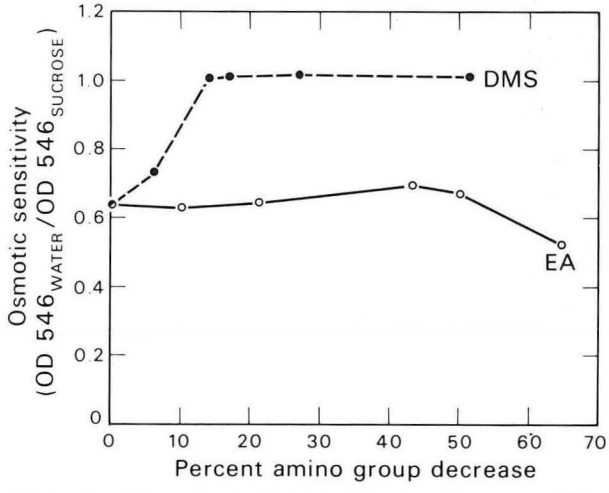

Fig. 4. Osmotic sensitivity of amidinated mitochondria. Mitochondria were amidinated as described in Fig. 2 and in Methods. Aliquots (0.5–1.0 mg protein) were added to 1.0 ml either deionized water or 0.25 M sucrose. After 10 min of equilibration at room temperature, the optical density of the mitochondrial suspensions was read at 546 nm. Osmotic sensitivity is expressed empirically as the ratio, OD 546 (H_2O)/OD 546 (sucrose). ●- - - -●, DMS; ○——○, EA.

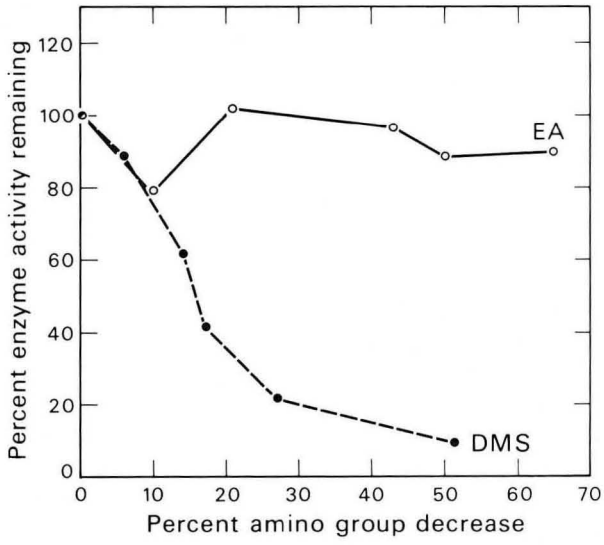

Fig. 5. The effect of amidination on mitochondrial electron transport. Amidinated mitochondria were prepared as described in Fig. 2 and in Methods. Ascorbate-TMPD oxidase activities were determined polarographically at room temperature as described in Methods. Activities are given as percent of untreated controls. ●----●, DMS; ○——○, EA.

variation was noted in the extent of inhibition associated with different degrees of amidination. However, within the same mitochondrial preparation, the relative inhibitory capacity of mono- and bifunctional imidates was highly reproducible.

DISCUSSION

This report describes the use of imidates to chemically modify rat liver mitochondria. These reagents have been shown previously to modify soluble protein with minimal perturbation of structure (6, 16). The present study has demonstrated that these reagents can interact with more complex systems, such as mitochondria, in an innocuous manner. More than 60% of free amino groups of this organelle can be amidinated by a monofunctional imidate, EA, with little effect on ascorbate-TMPD oxidase activity. Monofunctional imidates have also been shown to react mildly with erythrocytes (4) and photoreceptor membranes (17). At low concentrations (1–5 mM) the bifunctional reagent, DMS, also proved to be a mild alkylating agent. Although mitochondria treated with these concentrations of DMS fail to respond osmotically and display extensive polypeptide crosslinking, these modified organelles possess 60–90% of ascorbate-TMPD oxidase activity of untreated controls. One particularly interesting application utilizing the mild nature of amidination involves the use of another bifunctional imidate, dimethyl adipimidate, to modify human sickle erythrocytes. Although these treated cells do not display hypoxia-induced sickling, they show little alteration of both rheological and metabolic properties (18).

At higher concentrations (10–50 mM), DMS was an effective inhibitor of ascorbate-

TMPD oxidase activity. This inhibition cannot be attributed to amidination directly, since monofunctional imidates had little effect on activity even at very high levels of amidination. Furthermore, considering the extent of monofunctional reaction, it is unlikely that DMS reacts with amino groups other than those complexed by EA and MBI. Since DMS is capable of bifunctional amidination, the inhibition observed may be due to the cross-linking property of this reagent. The crosslinking reaction in membrane systems is a complex process involving protein-protein, protein-lipid, and lipid-lipid coupling. Protein-lipid crosslinking has been demonstrated in DMS treated erythrocyte membrane (19). Furthermore, intramolecular crosslinking in protein may also occur. Crosslinking, irrespective of mode, would be expected to reduce molecular motion of components. For example, glutaraldehyde treatment has been shown to prevent the pH-induced aggregation-disaggregation cycle of erythrocyte membrane particles revealed by freeze-fracture electron microscopy (20). Furthermore, we have demonstrated that glutaraldehyde treatment of mitochondria causes a reduction in the motion of exogenous spin labels in the membrane hydrophobic domain (1).

Treatment of intact mitochondria with DMS also resulted in the crosslinking of matrix polypeptides. Although the mechanisms are unclear, it is possible that this phenomenon contributes to the inhibition of electron transport by DMS. To circumvent these problems we have extended our studies to sonically prepared isolated membranes. Preliminary experiments using this system have afforded similar results to those obtained with intact organelles. Our results, therefore, suggest that crosslinking may inhibit function by restricting motion between integrated membrane components and allude to the possible involvement of molecular motion in the proper functioning of the terminal end of the mitochondrial electron transport chain.

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