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PHOTODYNAMIC ACTION OF BILIRUBIN ON THE INNER MITOCHONDRIAL MEMBRANE. IMPLICATIONS FOR THE ORGANIZATION OF THE MITOCHONDRIAL ATPASE

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Summary. Bilirubin in the presence of O_2 and light catalyzes the photodynamic modification of the proteins of the inner mitochondrial membrane as monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Numerous polypeptide bands become streaked towards higher apparent molecular weight and decrease in staining intensity while other bands remain largely unchanged. The loss in staining intensity which occurs is at least partially due to apparent cross-linking of the polypeptides to produce aggregates which cannot penetrate into the gel. The α and β bands of the mitochondrial ATPase differ markedly in their susceptibility to modification. The β subunit is rapidly modified while the α subunit is largely inert. This differential susceptibility is a consequence of the binding of the soluble F_1 ATPase to the membrane. When submitochondrial particles with their normal complement of bound F_1 are mixed with free F_1 and are photolyzed together in the presence of bilirubin and O_2, it is found that the β subunit of the membrane-bound F_1, but not the α subunit, has been modified while neither subunit of the free F_1 has been modified. This increased susceptibility of the β subunit in the membrane state may represent cross-linking to membrane components and is consistent with the β subunit making more extensive contacts with membrane components than does the α subunit.

Bilirubin and other photosensitizers in the presence of O_2 have been shown to catalyze the photodynamic modification of the red blood cell membranes. This complex and not well understood series of reactions results in the peroxidation of membrane lipids and the modification of membrane proteins. Destruction of amino acid side chains is observed (1) as is aggregation of intramembraneous particles as seen in freeze-fracture electron microscopy (2) and cross-linking of membrane proteins as monitored by SDS-PAGE[1] (2-4). Pronounced differences are observed in the susceptibility of the proteins to cross-linking. It is of interest to determine the nature of those photodynamic reactions, both to obtain a better understanding of the reactions themselves and in order to evaluate the possible usefulness of the differences in susceptibility for the characterization of the organization of membrane proteins.

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[1]Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; OSCP, oligomycin sensitivity conferring protein.

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The present report demonstrates that bilirubin catalyzes the photodynamic modification of the proteins of the inner mitochondrial membrane as monitored by SDS-PAGE. The differences in susceptibility of the proteins to modification is striking, particularly for the proton translocating ATPase. This enzyme has two principle domains (see 5 for review); a soluble component, F_1, which can be readily stripped from the membrane and an intergral membrane component, F_o. The F_1 is composed of five types of polypeptide chains designated α, β, γ, δ and ε in order of size. The β subunit contains the active site for ATP hydrolysis and presumably ATP synthesis as well. The F_o component contains a proton pore through the membrane. On photolysis, the β subunit of the F_1 ATPase is more rapidly modified than the α subunit, but only when F_1 is membrane-bound and not when free. This result has implications for the organization of the complete membrane-bound ATPase.

METHODS

Preparations. Submitochondrial particles (Type ETPH (Mg^{2+},Mn^{2+})) were prepared from beef heart mitochondria by the method of Beyer (6). The final pellet was homogonized in 250 mM sucrose, 10 mM Tris-HCl pH 8.0 and stored at -20°C. The F_1 ATPase was released from the membrane by treatment with chloroform (7). Crude F_1 was obtained by ammonium sulfate precipitation and purified F_1 was obtained by the procedure of Spitsberg and Blair (8). Protein was determined by the procedure of Lowry (9).

Photolysis. A bilirubin (Sigma Chem. Co.) stock solution was prepared with a final pH of 8.1 - 8.3 by dissolving bilirubin with 2 equivalents of NaOH and diluting to final volume with 250 mM sucrose, 10 mM Tris-HCl, pH 7.6. Submitochondrial particles were diluted to 5 mg/ml with 250 mM sucrose, 10 mM Tris-HCl, pH 8.0, which had been saturated with oxygen by bubbling a stream of O_2 through it. An aliquot of the bilirubin solution was added with stirring to a final concentration of 0.5 mM. Photolysis was performed in 1 ml batches with stirring in a 10 ml beaker. The temperature was maintained at 0°C by immersion of the beaker in ice water, except for photolysis with free F_1, which was performed at 25°, in a circulating water bath. The light source was a 650 watt DVY type tungsten-halogen lamp located 25 cm above the sample. The light was filtered through a 1.2 cm layer of 4% $CuSO_4$ in a Pyrex evaporating dish. During photolysis a stream of moist O_2 was directed over the stirred surface of the sample.

SDS-PAGE. Electrophoresis was performed by the procedure of Laemmli and Favre (10) except that the SDS concentration in the gel and running buffer was increased to 0.2%. The acrylamide concentration was 12% in the separating gel and 5% in the stacking gel. Gels were stained overnight with Comassie Blue R-250 (Sigma Chem. Co.) and destained with 10% acetic acid, 10% isopropanol.

RESULTS AND DISCUSSION

The effects on submitochondrial particles of irradiation in the presence of bilirubin plus oxygen was determined by analysis of the change in the pattern of polypeptides observed by SDS-PAGE. Results of a typical experiment are given in Figure 1 which gives both a standard gel and one which has been run for twice as long to give better resolution of the high molecular weight polypeptides. Identical control patterns are obtained for untreated particles,

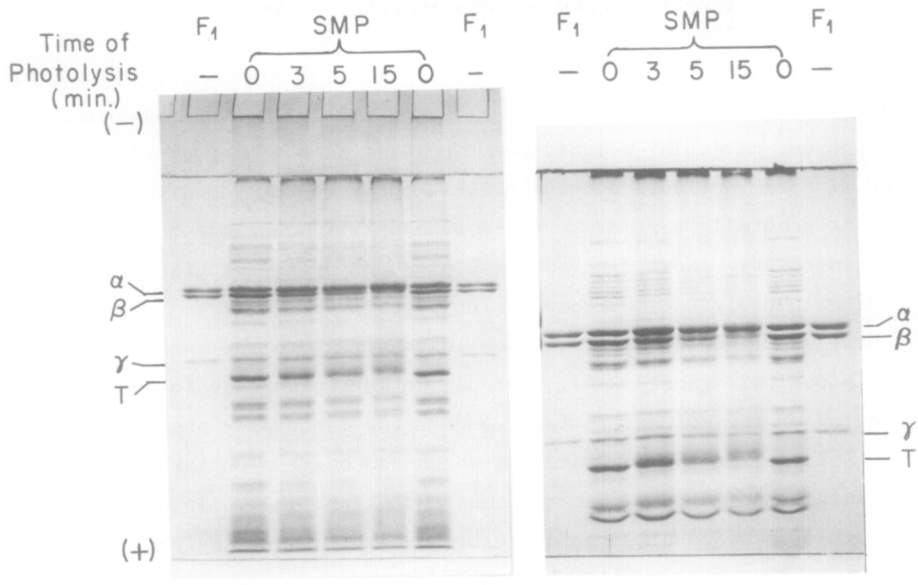

Figure 1. Effect of photolysis on SDS-PAGE of submitochondrial particles. Photolysis with bilirubin and O_2 performed as described in Methods. The large subunits of F_1 are designated α, β, and γ; T is the adenine nucleotide translocase.

ones treated with bilirubin plus oxygen in the dark and particles exposed to light in the presence of oxygen but without bilirubin. The α, β and γ subunits of the mitochondrial ATPase, F_1, can be identified by co-migration with the subunits of purified F_1. Submitochondrial particles which have been selectively stripped (11) of the soluble F_1 component by extraction with 2-3 M urea showed greatly reduced staining at the position indicated for α, β and γ although other bands were unaltered. The major hydrophobic protein which is the adenine nucleotide translocase (12) can be identified by its molecular weight, high concentration and resistance to solubilization by chaotropic agents.

On exposure to light, the pattern changes markedly. There is a general loss of resolution and streaking of many bands with an increase in the background staining throughout the whole track. This streaking occurs in such a way that the average position of many bands shifts towards the origin. This behavior is most clearly demonstrated by the nucleotide translocase, but can also be observed with other bands. One interesting possibility is that this apparent increase in molecular weight and streaking represents the covalent attachment of a variable number of lipid molecules to the membrane polypeptides. A second class of polypeptides is examplified by the α subunit of F_1 which shows no shift in position or streaking, but does show a small decrease in staining intensity. This lack of modification is consistent with the α subunits occupying a position which is far removed from the integral regions of the

membrane and the lipid peroxidation and side reactions, which accompany photolysis. The β subunit of F_1, however, is readily modified and is lost from its initial position on the gel. There is some streaking of the β subunit to higher apparent molecular weight, but the shift is not as pronounced as that observed with the nucleotide translocase. The γ subunit does not change position and is generally similar to the α although it does lose relatively more staining intensity. Some discreet, new bands do appear at high molecular weight after photolysis, but they are only weakly stained.

With submitochondrial particles in constrast to the results obtained with red blood cell ghost (2-4), there is no large increase in the staining near the top of the gel and in fact, a decrease is usually observed. There is, however, a large increase in material which can be seen on the surface of the 5% acrylamide stacking gel, but does not photograph well. In order to confirm the suspected presence of large aggregates, samples were analyzed by gel filtration in SDS. As shown in Figure 2, there is a large increase in high molecular weight material after photolysis as indicated by the increased peak at the void volume (fraction 12). Analysis of the column fractions by SDS-PAGE indicates that the material at the void volume runs at the stacking-separating gel interface before photolysis, but cannot penetrate the stacking gel after photolysis and can be seen on the surface of the gel. Apparently, most of the cross-linked product with submitochondrial particles is so large that it cannot penetrate into the stacking gel. The α and β subunits reach

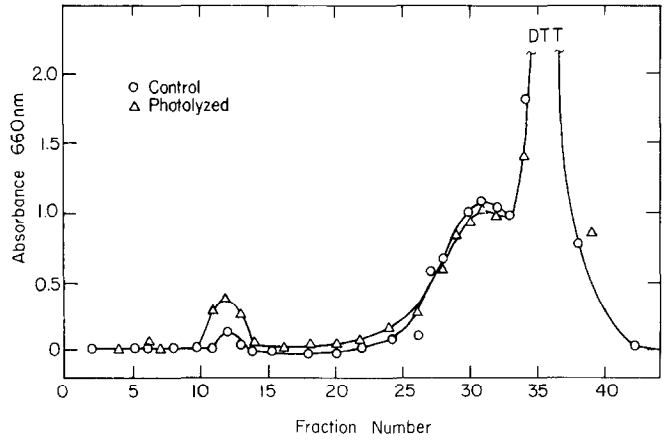

Figure 2. Gel filtration in SDS on Sepharose CL-2B of photolyzed and unphotolyzed submitochondrial particles. One ml of 5 mg/ml submitochondrial protein (before or after photolysis) was centrifuged and the pellet dissolved in one ml of 25 mM potassium phosphate, pH 7.5, 0.1 mM EDTA, 3% SDS and 15 mM dithiothreitol (DDT). The solution was heated to 100°C for 2 min. and then chromatographed on a 1.6 x 25 cm column of Sepharose CL-2B. Column buffer was 25 mM potassium phosphate, pH 7.5, 0.1 mM EDTA and 1% SDS. Protein was determined by Lowry (9) and reported as A_{660}.

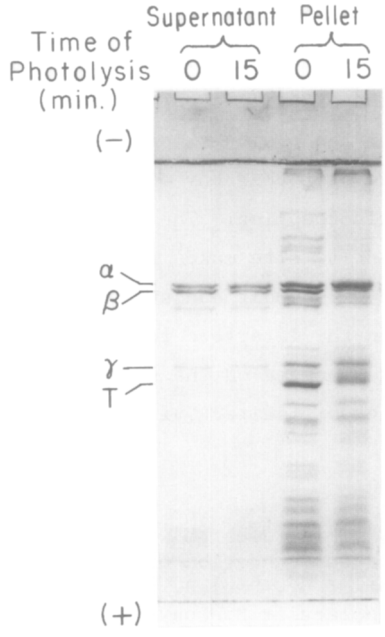

Figure 3. Effect of photolysis on SDS-PAGE of submitochondrial plus free F_1. Particles and free F_1 were separated by centrifugation and analyzed separately.

maximum concentration in fractions 28-30 and show both α and β in the unphotolyzed samples, but only α in the photolyzed ones.

The shift of many bands to higher apparent molecular weight was not noted in the work with the red blood cell membranes (3,4), but this could be due to the use of tube gels as opposed to the slab gels used here which allow a more accurate, side-by-side, evaluation of small differences in mobility. Also the present study employed a stacking buffer system which produces sharper bands.

In order to evaluate any selective effect on the β subunit which was not a consequence of its membrane attachment, a sample of submitochondrial particles was supplemented with soluble F_1 and photolyzed as before with bilirubin in the presence of oxygen. After photolysis, the soluble F_1 and membranes were separated by centrifugation and analyzed separately by SDS-PAGE as shown in Figure 3. The F_1 attached to the membrane has undergone preferentia loss of the β subunit analogous to the results seen in Figure 1 while the free F_1 is largely unaffected and maintains a constant α : β ratio. The bands present in the supernatent in addition to those due to F_1 subunits were present in the added F_1 preparation which had not been purified by ion-exchange chromatography. No proteins were observed in control experiments to be released from the membrane by the photolysis procedure.

Implications for Organization of Mitochondrial ATPase. The proton translocating ATPase of the mitochondrial membrane is one of the most complex enzymes known. One of the major unsolved problems in bioenergetics is the nature of the coupling of the proton transport through F_o to the synthesis of ATP by F_1 and a knowledge of topography of the complete enzyme will be important in understanding this coupling. The results reported here indicate that the β subunit with its active site makes more extensive contact with membrane components than does the α subunit and support a model in which at least some region of β is located in the interface region between F_1 and the membrane while the α subunits are located on the side of F_1 away from the membrane. Such contact may be direct or indirect via other peripheral proteins such as OSCP or F_6.

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