LABELING OF COMPLEX III PEPTIDES IN BEEF HEART MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES BY DIAZONIUM BENZENE [^{35}S]SULFONATE

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1. Introduction

The transmembrane distribution of mitochondrial proteins has been previously studied with the non-penetrating, protein reagent, *p*-diazonium benzene sulfonate (DABS). Different patterns of protein labeling were observed in both rat liver [1] and beef heart [2,3] mitochondria when either the matrix or cytoplasmic surfaces of the inner membrane were exposed to DABS. The identities of the labeled peptides are not known. It has been reported [1,4] that electron transport activity is specifically inhibited in the cytochrome b–c_1 region of DABS-treated mitochondria, possibly at the site of the oxidation factor [4]. In keeping with the effect of DABS on the cytochrome b–c_1 region, we have shown that DABS inhibits duroquinol–cytochrome *c* reductase activity in isolated Complex III [5], but it apparently does not inhibit enzyme activity of isolated cytochrome oxidase [6]. Inhibition of duroquinol-cytochrome *c* reductase is, however, accompanied by the labeling of nearly all the peptides of isolated Complex III [5].

In the present paper we report the labeling of Complex III peptides in DABS-treated mitochondria and submitochondrial particles from beef heart. Our results are consistent with the labeling of the two core proteins [7,8] on the matrix surface of the inner membrane, and of a 29 000 molecular weight peptide on the cytoplasmic surface.

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2. Methods

Beef heart mitochondria, EDTA-submitochondrial particles [9], Complex III [10], and F_1-ATPase [11] were prepared as described.

[^{35}S]DABS was prepared from [^{35}S]sulfanilic acid (Amersham) by the method of DePierre et al. [12]. Protein labeling was carried out for 30 min at 0–4°C in a medium containing: 1–2 mg protein/ml, 0.25 M sucrose, and 1.5 mM DABS (60–90 mCi/mmol). The reaction was stopped by addition of 72 mM histidine, and the protein was removed by centrifugation. The pellet was washed once in a buffer containing 50 mM Tris–HCl, pH 7.5, and 0.25 M sucrose, and was suspended in 38 mM Tris–glycine, pH 8.6. Complex III and F_1-ATPase were removed from the incubation mixture by precipitating with 50% saturated ammonium sulfate (0°C). The final pellet was dissolved in 2% dodecyl sulfate (SDS), 5% mercaptoethanol, and 10 mM Tris–HCl, pH 7.5.

Rocket immunoelectrophoresis was performed on DABS-labeled membranes as described [13]. The precipitation lines formed against Complex III antibodies [13] were cut from the agarose gels and extracted with 2% SDS, 5% mercaptoethanol and 10 mM Tris–HCl, pH 7.5. The extracted proteins were electrophoresed on polyacrylamide, gradient pore gels (10–20% polyacrylamide), containing SDS in the system of Laemmli [14]. The gels were stained with coomassie blue, dried, and exposed to X-ray film for localization of radioactive peptides.

3. Results

Figure 1 shows coomassie blue staining (fig.1B) and autoradiographs (fig.1A) of mitochondrial and submitochondrial membranes labeled with [^{35}S]DABS. For comparison the labeling patterns of isolated Complex III and F_1-ATPase are also shown. In agreement with results reported for rat liver material [1], the labeling pattern of beef heart mitochondria and submitochondrial particles are different. In DABS-treated mitochondria, radioactivity is located predominantly in three peptides of 29 000, 44 000, and 70 000 molecular weight. Several minor bands are also present. Peptides corresponding to 29 000 and 70 000 molecular weight are also labeled in isolated Complex III, whereas the 44 000 molecular weight peptide is not. The latter peptide is not one of the core proteins of Complex III as seen by its slightly higher mobility on SDS gels (fig.1).

In contrast to mitochondria, labeling of submitochondrial particles is characterized by a large number of more weakly labeled bands (fig.1A). The major peptide labeled in submitochondrial particles has a molecular weight on SDS-polyacrylamide gels of 29 000. The molecular weights of the other heavily labeled peptides (50 000 and 47 000) correspond

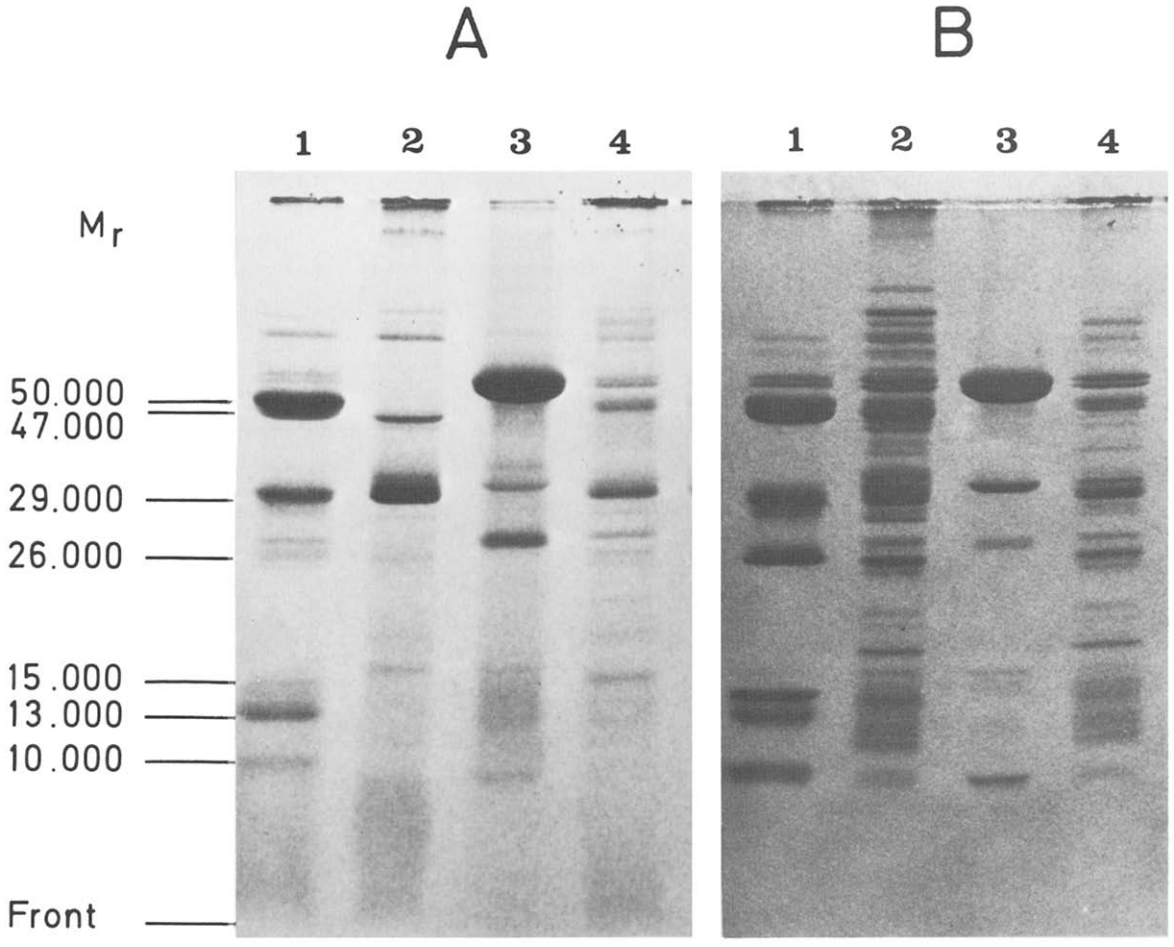

Fig.1. Incorporation of ^{35}S-diazonium benzene sulfonate into the peptides of mitochondria and submitochondrial particles. Autoradiographs (A) and coomassie blue staining (B) of: (1) isolated Complex III, (2) mitochondria, (3) F_1-ATPase and (4) submitochondrial particles.

exactly to the two core proteins of Complex III [7,8]. As expected, the α and β subunits of F_1-ATPase are labeled only in submitochondrial particles (fig.1A) indicating that DABS does not penetrate the membrane to a significant extent during the incubation. As reported earlier [8], F_1-ATPase can appear as a contaminant in Complex III (fig.1).

In contrast to the results of Eytan et al. [2], we see no evidence of labeled peptides in intact mitochondria which correspond in molecular weight to subunits V and VII of cytochrome oxidase. Also in our experiments, a peptide of 70 000 molecular weight is heavily labeled in both Complex III and mitochondria, but not in submitochondrial particles. In Complex III this peptide is thought to be a contamination from the large subunit [15] of succinate dehydrogenase [8]. This raises a general problem, exemplified here with the 70 000 component in mitochondria, that the labeled bands on SDS-polyacrylamide gels could be composed of multiple peptides.

The experiments reported above (fig.1) suggest that the major peptide labeled on the cytoplasmic surface of the inner membrane has a molecular weight of 29 000 and corresponds to one of the peptides of isolated Complex III. Labeling of the matrix surface, on the other hand, labels two peptides which correspond to the core proteins of Complex III as well as a peptide of 29 000. To test if these peptides are components of Complex III, DABS-labeled mitochondria and submitochondrial particles were immunoprecipitated with antibodies to Complex III, and the immunoprecipitates were electrophoresed and exposed to film. The results of these experiments are shown in fig.2. It is quite clear that the core proteins

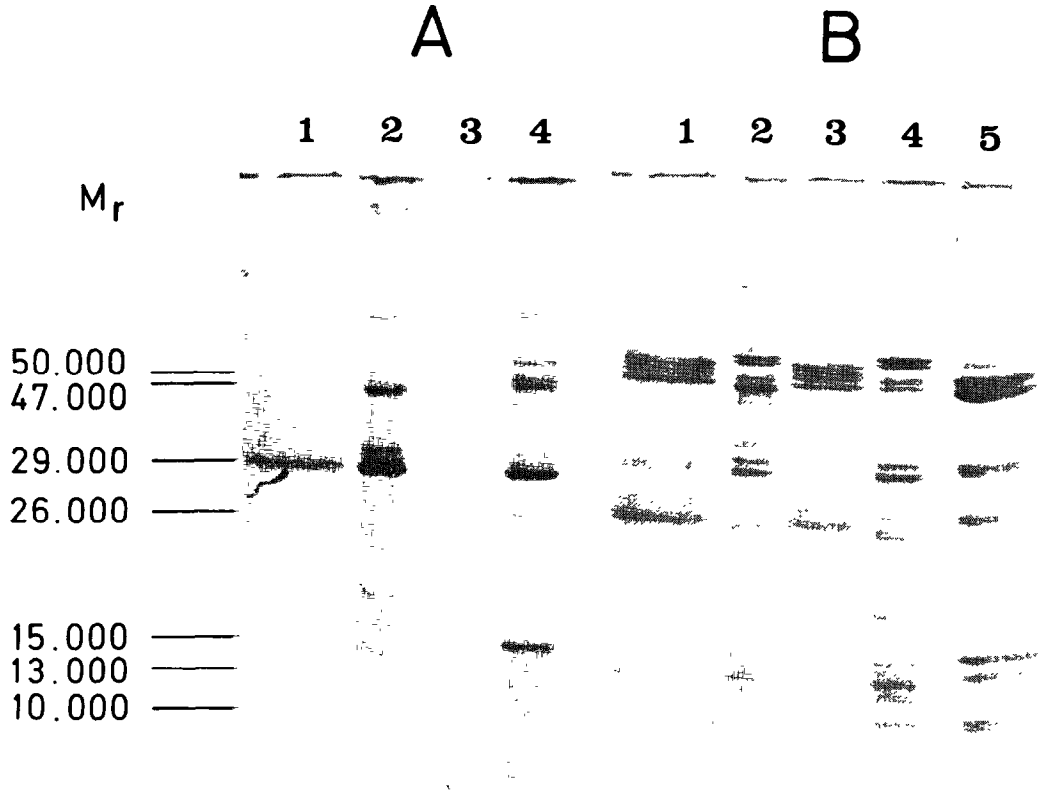

Fig.2. Incorporation of ^{35}S-diazonium benzene sulfonate into the peptides of Complex III. Autoradiographs (A) and coomassie blue staining (B) of: (1) Complex III immunoprecipitated from mitochondria, (2) mitochondria, (3) Complex III immunoprecipitated from submitochondrial particles, (4) submitochondrial particles and (5) Complex III.

are labeled only from the matrix side of the membrane. The 29 000 molecular weight peptide is most heavily labeled from the cytoplasmic side of the inner membrane, but weak labeling of this peptide is also observed in immunoprecipitates from submitochondrial particles.

4. Discussion

The present study provides evidence that the core proteins of Complex III [7,8] are accessible to DABS only from the matrix surface of the mitochondria, since labeling of the peptides was observed in submitochondrial particles but not in intact mitochondria. This asymmetry of labeling, plus the finding that the α and β subunits of F_1-ATPase were labeled only in submitochondrial particles, strongly suggests that DABS did not penetrate the membrane to a significant extent during the incubation [see also 1–3,6].

When mitochondria are labeled from the cytoplasmic surface, Complex III is labeled primarily in the peptide of 29 000 molecular weight. It has been suggested [16] that this region of the SDS gel contains peptides of both cytochrome c_1 and cytochrome b. Since cytochrome b is thought to be a very hydrophobic protein [17] it is tempting to suggest that the peptide exposed to DABS is cytochrome c_1. The heme-binding peptide of cytochrome c_1 is thought to be exposed on the cytoplasmic surface of the inner membrane [18]. Such an assignment must, however, await more reliable information concerning the identity of the peptides in this region of the SDS gel.

Intact mitochondria labeled with lactoperoxidase and ^{125}I are also labeled primarily in a peptide of 29 000 molecular weight [19,20]. This peptide is identical with the carboxyatractyloside-binding protein of the ATP/ADP translocator [20,21]. To our knowledge, no experiments have been reported showing that the carboxyatractyloside-binding protein is labeled with DABS in intact mitochondria, although the labeling patterns obtained with DABS [1, fig.1] and lactoperoxidase + ^{125}I [19,20] are very similar. It should be mentioned, however, that the 29 000 molecular weight peptide is relatively heavily labeled in isolated Complex III treated with DABS [5], whereas very little label is found in this peptide after treating isolated Complex III with lactoperoxidase and ^{125}I [5].

Treatment of intact mitochondria with DABS leads to inhibition of electron transport in the cytochrome b–c_1 region [1,4]. In keeping with this observation, we have reported that DABS inhibits duroquinol–cytochrome c reductase activity in isolated Complex III [5]. Isolated cytochrome oxidase is apparently not effected by treatment with DABS [6]. Grigolova and Konstantinov [4] postulated that inhibition of electron transport in the cytochrome b–c_1 region of DABS-treated mitochondria might be due to interference with the oxidation factor. The latter is a protein needed for electron transport between cytochrome b and cytochrome c_1 [21], and whose molecular weight has been reported as 15 000 [16]. In our experiments, however, no labeling of a mitochondrial peptide corresponding to 15 000 molecular weight has been observed. An alternative explanation for the inhibition pattern produced by DABS treatment of mitochondria [4] could be that DABS modifies the heme-binding peptide of cytochrome c_1 directly (fig.1), and not the oxidation factor.

It should be stressed that experiments such as those reported here can only be used to establish the accessibility of a particular peptide to DABS. Lack of labeling does not constitute proof that a peptide is absent from the membrane surface which has been treated with DABS.

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References

[1] Tinberg, H. M., Melnick, R. L., Maguire, J. and Packer, L. (1974) Biochim. Biophys. Acta 345, 118–128.
[2] Eytan, G. D., Carroll, R. C., Schatz, G. and Racker, E. (1975) J. Biol. Chem. 250, 8598–8603.
[3] Schneider, D. L., Kagawa, Y. and Racker, E. (1972) J. Biol. Chem. 247, 4074–4079.

[4] Grigolova, I. and Konstantinov, A. (1977) FEBS Lett. 78, 36–40.
[5] Gellerfors, P. and Nelson, B. D. (1977) Eur. J. Biochem. 80, 275–282.
[6] Eytan, G. D. and Schatz, G. (1975) J. Biol. Chem. 250, 767–774.
[7] Silman, H. I., Rieske, J. S., Lipton, S. H. and Baum, H. (1967) J. Biol. Chem. 242, 4867–4875.
[8] Gellerfors, P. and Nelson, B. D. (1975) Eur. J. Biochem. 52, 433–443.
[9] Lee, C. P. and Ernster, L. (1967) Methods Enzymol. 10, 543–548.
[10] Rieske, J., Zaugg, W. S. and Hausen, R. E. (1964) J. Biol. Chem. 239, 3023–3030.
[11] Horstmann, L. L. and Racker, E. (1970) J. Biol. Chem. 245, 1336–1344.
[12] DePierre, J. W. and Karnovsky, M. L. (1974) J. Biol. Chem. 249, 7111–7120.
[13] Nelson, B. D. and Mendel-Hartvig, I. (1977) Eur. J. Biochem. 80, 267–274.
[14] Laemmli, U. K. (1970) Nature 227, 680–685.
[15] Davis, K. A. and Hatefi, Y. (1971) Biochemistry 10, 2509–2516.
[16] Marres, C. A. M. and Slater, E. C. (1977) Biochim. Biophys. Acta 462, 531–548.
[17] Weiss, H. (1976) Biochim. Biophys. Acta 456, 291–313.
[18] Schneider, D. C. and Racker, E. (1971) Fed. Proc. 30, 1190.
[19] Boxer, D. H. (1975) FEBS Lett. 59, 149–152.
[20] Brdiczka, D. and Schumacher, D. (1976) Biochem. Biophys. Res. Commun. 73, 823–832.
[21] Boxer, D. H., Feckl, J. and Klingenberg, M. (1977) FEBS Lett. 73, 43–46.
[22] Nishibayashi-Yamashita, H., Cunningham, C. and Racker, E. (1972) J. Biol. Chem. 247, 698–704.