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HYDROPHOBIC PHOTOLABELLING OF THE YEAST CYTOCHROME *c* OXIDASE SUBUNITS IN CONTACT WITH LIPIDS

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Summary

The hydrophobic domain of the membrane-bound enzyme yeast cytochrome *c* oxidase was labelled with photoactivable phosphatidylcholines.

Subunits I, II and III were labelled; a minor labelling was also found on subunits V and VII.

The labelling of subunit V was located in a small terminal polypeptide sequence.

Cytochrome *c* oxidase isolated from *Saccharomyces cerevisiae* mitochondria is one of the best characterized eukaryotic oxidases [1]. This enzyme can be resolved by SDS-polyacrylamide gel electrophoresis into at least seven different subunits of apparent molecular weight ranging from 40 000 to 4500. The three larger subunits are coded by mitochondrial DNA, whereas the four smaller subunits are coded by nuclear genes [2,3]. The enzyme spans the membrane and most of its mass is exposed to the aqueous phase [4]. Subunits II, III, IV, VI and VII appear to possess hydrophilic domains since they are labelled by hydrophilic chemical reagents [5]. In order to identify the subunits forming the lipid-protein boundary Cerletti and Schatz [6] have used small photoactivable probes, which partition out predominantly in the lipid phase. Subunits I, II, III and VII were labelled and only a minor amount of radio-

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Abbreviation: SDS, sodium dodecyl sulphate.

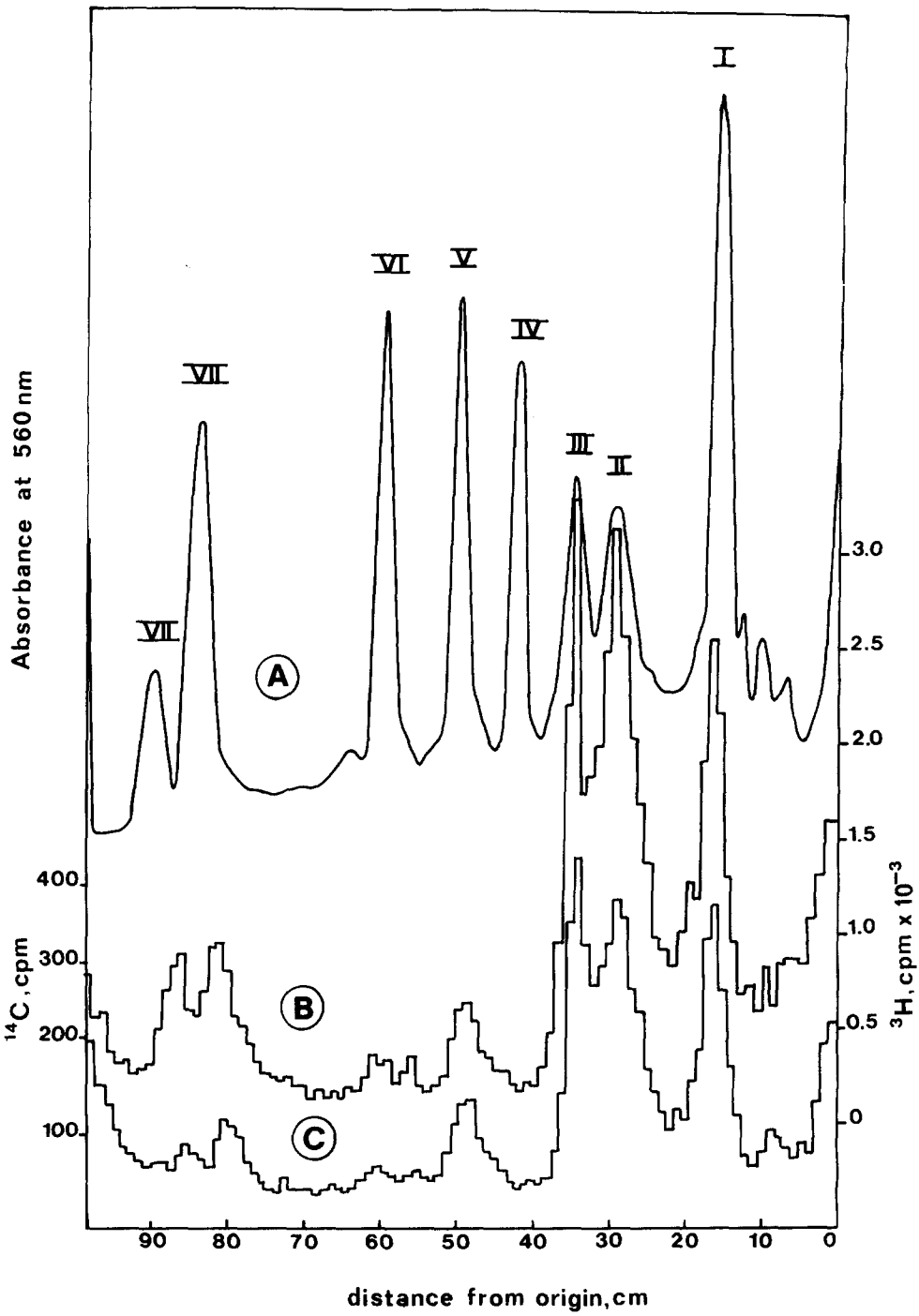

Fig. 1. Labelling of yeast cytochrome *c* oxidase with azidophospholipids. A. Densitometric trace of the Coomassie-Blue-stained gel. 50 μg of protein were subjected to electrophoresis as reported above. B and C. Distribution of radioactivities of phospholipid II (^{3}H) and phospholipid I (^{14}C), respectively, associated with the same gel.

activity was found at subunit IV and V. A similar kind of study was carried out independently on bovine heart cytochrome *c* oxidase using photoactive radioactive phospholipids bearing the photoactive group at two different levels of one fatty acid chain, which allows a more defined localization of the photoactive group in the lipid bilayer [7]. These probes label only the hydrophobic sector of membrane proteins [8,9]. The labelling of the hydrophobic sector of yeast cytochrome *c* oxidase with photoactive phospholipids is reported here in order to compare cytochrome *c* oxidases from different sources and the labelling patterns obtained with different probes.

Different batches of yeast cytochrome *c* oxidase were a kind gift of Professor G. Schatz. Enzymatic activity was measured according to Birchmeier et al. [10]. The preparation of 1-myristoyl, 2-12-amino-*N*(2-nitro-4-azidophenyl) dodecanoyl *sn*-glycero-3-phospho[^{14}C]choline (phospholipid I) and of 1-palmitoyl, 2-(2-azido-4-nitrobenzoyl) *sn*-glycero-3-phospho[^{3}H]choline (phospholipid II) is reported elsewhere [11]. Their specific radioactivities were, respectively, 177 mCi/mmol and 3.1 Ci/mmol. Phospholipids I and II were incubated with the enzyme as previously reported [7]. Alternatively they were dried at the bottom of a tube with a nitrogen flux and then pumped under vacuum for 2 h. 50 μl of deaerated 50 mM phosphate buffer, pH 7.2, 0.15 mM EDTA, 0.1% cholate or 1% octylglucoside were added to the tube and rotated for 1 h. 140 μg of enzyme were incubated with this mixture at 0°C for 1 h and then diluted to 750 μl with deaerated buffer without detergent. The sample was irradiated for 20 min at 0°C under nitrogen in a glass cuvette with a 100 W long-wave ultraviolet lamp (Ultra Violet, San Gabriel, CA, U.S.A.) and then layered on a 10% sucrose, 50 mM phosphate buffer pH 7.2 and centrifuged for 3 h at 224 000 × *g* in a Sorvall AH-650 rotor. The pellet was analyzed by electrophoresis in 15% polyacrylamide-SDS-urea slab gels according to Swank and Munkres [12]. Labelling of the enzyme under denaturing conditions was performed by preincubation in 10 mM phosphate buffer, pH 7.2, 0.15 mM EDTA, 2% SDS for 20 min at 50°C and then illuminated as before. Trypsin digestion was performed by incubating the enzyme with trypsin (10 μg/mg of oxidase) for 1 h at 25°C after ultraviolet irradiation. The reaction was stopped by adding trypsin inhibitor. In the control sample the inhibitor was added to the oxidase before trypsin. Radioactivity associated with the gel was determined as previously reported [7].

Among the several methods available for the reconstitution of protein-lipid complexes from isolated membrane proteins and exogenous lipids, we have found that the detergent dilution procedure of Racker et al. [13] allows a higher association of exogenous lipids with yeast cytochrome *c* oxidase. Both cholate and octylglucoside were effective in mediating the addition of added lipids. This procedure results in enzymatically active lipid-protein complexes, but does not produce, in our hands, incorporation of the oxidase into vesicles in an uncoupler-dependent fashion. Fig. 1 shows the labelling profile of such a lipid-protein complex containing a trace (less than 0.5% w/w of total lipids) of the two photoactive phospholipids used in this study. Upon ultraviolet irradiation, the photoactive nitroarylazido group bound to the phospholipid molecule is converted into a reactive nitrene intermediate, which is able to cross-link the phospholipid to its neighbour molecules, thereby

labelling them radioactively [11]. Ultraviolet irradiation of yeast cytochrome *c* oxidase in the absence of oxygen does not affect its enzymatic activity. Most of the radioactivity in panels B and C of Fig. 1 is found at the gel front, where unbound phospholipids migrate. In fact, the same peak is found on gels of non-irradiated samples. While no radioactivity binds to non-irradiated

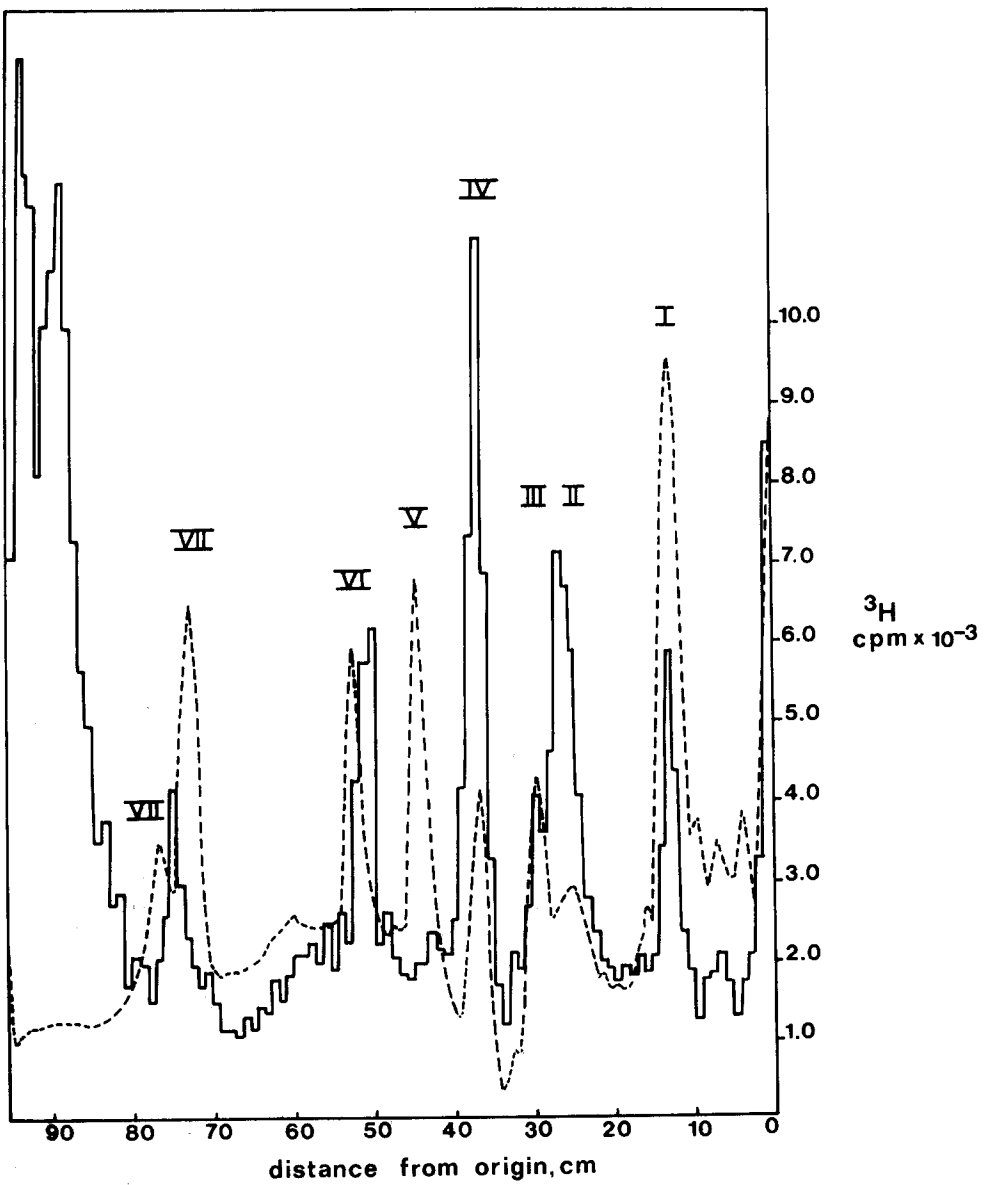

Fig. 2. Labelling of yeast cytochrome *c* oxidase under denaturing conditions. 50 μg of enzyme were labelled in the presence of SDS as reported above and then subjected to electrophoresis. The dashed line represents the Coomassie Blue densitometric profile, the continuous line reports the distribution of radioactivity along the gel.

protein, when yeast cytochrome *c* oxidase is illuminated subunits I, II and III are labelled. Minor amounts of labelling are found with subunits V and VII. Our electrophoretic conditions are able to resolve subunit VII of yeast cytochrome c oxidase into two components. This is consistent with the findings of Steffens et al. [14], showing that bovine heart cytochrome *c* oxidase subunit VII is composed by three polypeptides with different amino acid termini. The fact that the protein is labelled by azidophospholipids indicates that an exchange of phospholipids can occur at the surface of the hydrophobic sector of this membrane-bound enzyme. Moreover, it shows that the protein surface exposed to lipids comprises at least part of subunits I, II, III, V and VII. The same labelling pattern is obtained when the enzyme-lipid complex is formed by incubation with sonicated egg lecithin, tagged with photoactive phospholipids as previously reported [7].

The lack of labelling of subunits IV and VI may result from their non-

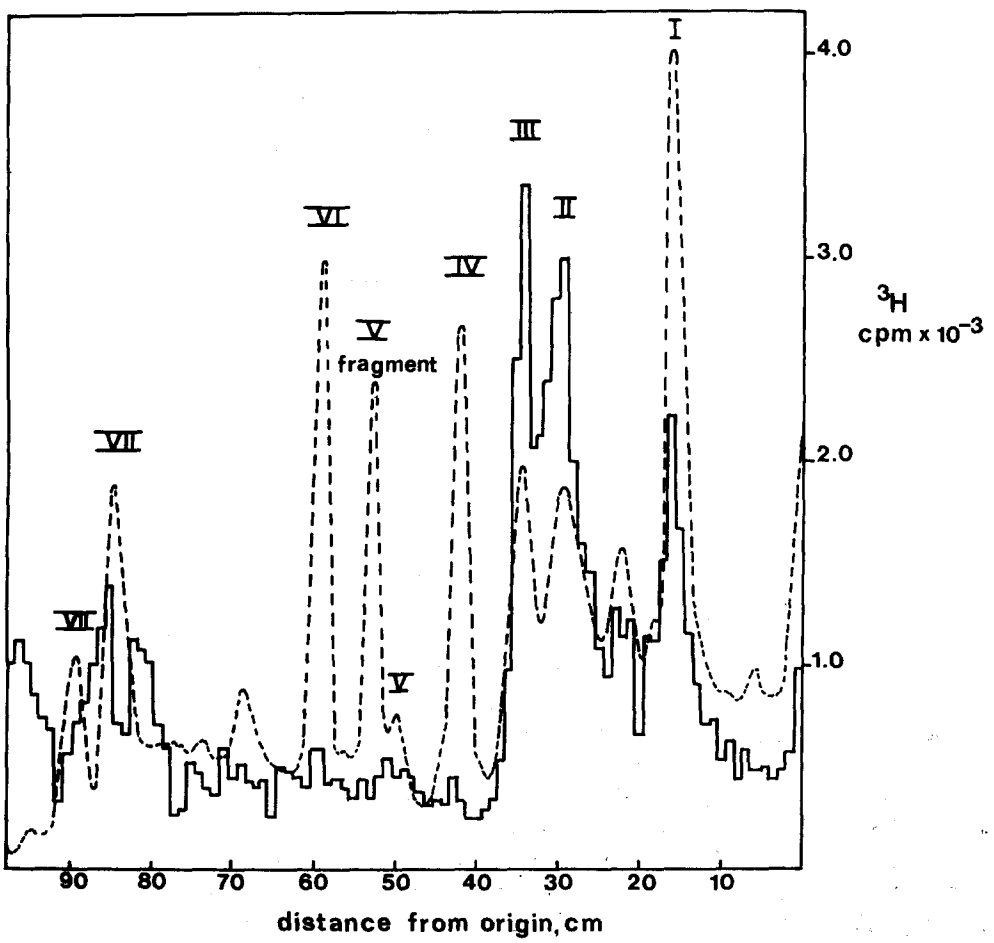

Fig. 3. Trypsin treatment of labelled yeast cytochrome c oxidase. The full line represents the distribution of radioactivity along the gel of a sample (50 μg) of protein treated with trypsin as reported above. The dashed line represents the densitometric profile of the gel after staining with Coomassie Blue.

interaction with phospholipids or from the inability of the photogenerated nitrene to react with them. However, Fig. 2 shows that also subunits IV and VI can be labelled under suitable conditions.

Trypsin treatment of yeast cytochrome *c* oxidase enhances its enzymatic activity (Schatz, G., personal communication). Moreover, this treatment has been shown by Prochaska et al. [15] to selectively remove some component from the gel electrophoretic pattern of bovine heart cytochrome *c* oxidase. The densitometric trace reported in panel A of Fig. 3 shows that trypsin splits specifically subunit V of yeast cytochrome *c* oxidase into a small and a large fragment, the latter having an apparent molecular weight intermediate between those of subunits V and VI. While the labelling of subunits I, II, III is not affected by trypsin treatment, no radioactivity is associated with the larger fragment of subunit V. This indicates that the hydrophobic labelling of subunit V is restricted to a small terminal portion of its polypeptide chain.

Lipid protein interactions are essential for the anchoring of membrane-bound enzymes to the lipid bilayer and for their enzymatic activity. The use of photoactive radioactive phospholipids, introduced by Chakrabarti and Khorana [16], is a direct chemical method to probe this interaction. In this study we have used this approach as a method for identifying which subunits of the cytochrome *c* oxidase from baker's yeast mitochondria form the lipid-protein boundary. The fact that the protein is labelled by arylazidophospholipids under conditions that fully preserve enzymatic activity demonstrates that added phospholipids are able to exchange with boundary lipids. This observation parallels that obtained by Jost et al. [17] on bovine cytochrome *c* oxidase with spin-labelled phospholipids. It appears that most of the surface of the hydrophobic sector of this enzyme is made up of the mitochondrially coded three larger subunits. A minor portion seems also to be contributed by subunit V and by subunits VII. Trypsin treatment of yeast cytochrome *c* oxidase leads to the specific removal of a small fragment from subunit V. No radioactivity is associated with the larger subunit V fragment, indicating that its hydrophobic peptide segment is very small and is situated in a terminal position.

Subunits IV and VI appear not to be accessible to the photoactive phospholipids used in the present study. This may be due to their external location with respect to the lipid bilayer or to the shielding effect of the other subunits. The first interpretation is in agreement with their being labelled by hydrophilic probes [5]. The same pattern of labelling has been obtained with both the detergent dilution and the lipid incubation procedures. Hence the labelling profile is not the result of the particular reconstitution procedure used.

Our results are thus essentially in agreement with those of Cerletti and Schatz [6] obtained with small photoactive hydrophobic probes. However different results are obtained with these two types of approach when applied to other membrane proteins such as the sodium, potassium-activated ATPase [18,19]. The similarity of the labelling of the protein subunits of the two oxidases does not imply that the same peptide segments, within a subunit primary sequence, are labelled. We are currently analysing which peptide segments of the subunits of the bovine heart cytochrome *c* oxidase, of

which the amino acid sequence is already available, are labelled by azidophospholipids.

The comparison of the labelling profiles with azidophospholipids of baker's yeast and bovine heart oxidases shows that in the enzyme from both sources nearly 90% of the total radioactivity associated with the protein on the gel is bound to the three larger polypeptides and to subunits VII (the labelled impurities present on the bovine heart cytochrome *c* oxidase preparations are not taken into account). Minor differences in the labelling of individual subunits may be related to different amino acid composition of their hydrophobic segments or to the different degree of their exposure to lipids.

The present results suggest a very similar structural organization of the polypeptide chains to form the hydrophobic sector in the cytochrome *c* oxidases from bovine heart and baker's yeast.

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