Selective labeling of beef heart cytochrome oxidase subunit III with eosin-5-maleimide

Michele Müller and Angelo Azzi*

Medizinisch-chemisches Institut der Universität Bern, Bühlstrasse 28, CH-3012 Bern, Switzerland

Received 13 March 1985

Cytochrome c oxidase has been isolated from beef heart mitochondria and labeled with the fluorochrome eosin-5-maleimide (EMA) after pretreatment with mersalyl. On SDS-polyacrylamide gels, EMA fluorescence and absorption occurred at a single band corresponding to subunit III. Since only Cys 115 of the two cysteinyl residues of subunit III had been shown to be reactive towards water-soluble SH-reagents, it was concluded that this residue was the one labeled by EMA. The EMA/enzyme ratio was about 1. Gel filtration experiments have shown that upon treatment with dicyclohexylcarbodiimide, subunit III was loosened from the complex; this result suggests that the inhibitory effect of dicyclohexylcarbodiimide on the H^+-translocation activity may be related to such a phenomenon.

Cytochrome oxidase *Subunit III* *Eosin-5-maleimide* *Selective labeling* N,N-*Dicyclohexylcarbodiimide*

1. INTRODUCTION

Subunit III of cytochrome *c* oxidase is the second largest polypeptide of the whole complex (12 or 13 subunits) having an M_r of 29918, as calculated from the DNA-inferred amino acid sequence [1]. This polypeptide has been suggested to be involved in the H^+-translocation function of the enzyme based on the following evidence. Inhibition of the H^+-pump by DCCD has been found to be associated with a parallel labeling of subunit III [2] whose removal from the complex resulted also in the loss of the H^+-transfer activity [3,4]. DCCD was shown to bind in the subunit III of the beef heart enzyme to the glutamyl residue 90 [5]. This subunit also contains 2 cysteinyl residues (Cys 115 and 218), which, contrary to glutamyl 90 or the cysteinyl residues 196 and 200, were not evolutionarily conserved. Cys 115 of subunit III was shown to be located at the cytoplasmic face of the inner mitochondrial membrane and to be accessible to water-soluble SH-reagents such as iodoacetamide and dithionitrobenzoate [6]. On the other hand, it was not possible to label Cys 218 even after dissociation and partial denaturing of subunit III by SDS [7]. In order to obtain structural information on membrane-bound proteins, eosin derivatives were largely used. Eosin is a very useful reporter group, since it can be used as a chromophore, as a fluorophore, and because of its high extinction coefficient and its suitable excitation and emission bands.

Eosin derivatives have been used to label band 3 in erythrocyte membranes [8], $(Ca^{2+}$-$Mg^{2+})$-ATPase in sarcoplasmic reticulum [9], the coupling factor CF1 in spinach chloroplasts [10] and the ADP/ATP translocator in beef heart mitochondria [11,12]. Here we present a method for specific labeling of subunit III of cytochrome oxidase with EMA (fig.1). EMA was used to selectively modify the cysteinyl residue 115 of subunit III, the only one which was shown to be reactive towards water-soluble SH-reagents. The association of subunit III with cytochrome *c* oxidase was followed using the

* To whom correspondence should be addressed

Abbreviations: $C_{12}E_{10}$, polyoxyethylene 10 laurylether; DCCD, dicyclohexylcarbodiimide; EMA, eosin-5-maleimide

Published by Elsevier Science Publishers B.V. (Biomedical Division)

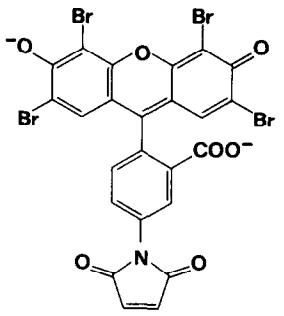

Fig.1. Molecular structure of eosin-5-maleimide.

eosin-labelled enzyme; it was found that binding of DCCD to the enzyme resulted in the loosening of subunit III.

2. MATERIALS AND METHODS

Bovine heart cytochrome oxidase was prepared as in [13]. The heme *a* content was calculated from spectra (dithionite-reduced minus air-oxidized) taken with an Aminco DW-2a spectrophotometer using a $\Delta\epsilon_{605-630nm} = 13.5\ mM^{-1}\cdot cm^{-1}$. EMA was dissolved in 100 mM sodium phosphate buffer (pH 7.2) and then diluted to 0.5 mg/ml (0.673 mM). The EMA concentration was calculated from spectra using a $\Delta\epsilon_{530-600nm} = 8.3 \times 10^{-4}\ M^{-1}\cdot cm^{-1}$ [14].

Cytochrome oxidase was labeled with EMA as follows: 1 nmol mersalyl was added to 1 nmol cytochrome oxidase in a buffer composed of 50 mM sodium phosphate (pH 7.2), 1% Na-cholate, and incubated for 30 min on ice; then 1 nmol EMA per nmol oxidase was added to the pretreated enzyme and incubated for 2 h on ice in the dark. The reaction was stopped by adding mercapto-1,3-propanediol in excess. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Kadenbach et al. [15], however without addition of bromphenol blue in the sample buffer.

Fluorographic pictures were obtained by photographing, through cut-off filters, the fluorescence emission of slab gels illuminated with UV light from below. An analysis of the same gel was performed using a special attachment for the Aminco DW-2a spectrophotometer [16] recording the EMA absorbance at the wavelength pair 530–600 nm. The slab gel was then fixed for 1 h with an aqueous solution composed of 25% ethanol and 14% formaldehyde [17]. Staining and destaining were performed conventionally using Coomassie brilliant blue.

Cytochrome *c* oxidase was labeled with DCCD (1 : 100 molar ratio) for 45 min at 20°C in the same buffer used for gel filtration experiments, composed of 75 mM glycine, 375 mM Tris-Cl, 0.5 M NaSCN, 0.1% Triton X-100, 0.1% $C_{12}E_{10}$ (pH 9.5) at 20°C. Gel permeation was performed on Fractogel TSK, HW-55(S) at 5.4 ml/h in a 1 × 30 cm column and fractions were collected each 15 min after the application of the sample (4–5 nmol enzyme). Protein content was determined using the method of Lowry et al. [17].

EMA was from Molecular Probes (Junction City, OR); mersalyl from Mann Res. Labs (New York); DCCD, Triton X-100 from Fluka AG (Buchs, Switzerland); $C_{12}E_{10}$ from Sigma (St. Louis, MO); Fractogel TSK HW-55(S) from Merck (Darmstadt, FRG).

3. RESULTS AND DISCUSSION

Fig.2A shows the results of the EMA labeling of cytochrome oxidase solubilized in 3% SDS. The labeling was performed at an EMA/oxidase molar ratio of 30, to ensure that the reagent would be in excess over the 17 cysteinyl residues per mol of the whole complex [19]. The absorbing or fluorescent bands of the molecular mass subunits could not be assigned to the known cytochrome *c* oxidase polypeptides because, after EMA labeling, a somewhat different electrophoretic pattern was observed. Since the electrophoretic migration of the largest subunits was not largely affected, an assignment in this case was possible. When the labeling was performed using the intact enzyme solubilized in cholate and pretreated with mersalyl, practically only subunit III was labeled (fig.2B,C). Additional high-molecular mass components labeled by EMA to a small extent may represent contaminants and/or small polypeptides aggregated with subunit III. The amount of unspecific label varied with the enzyme preparation. Additional gel filtration chromatography purification reduced the unspecific labeling to minor levels ($\leqslant$5%).

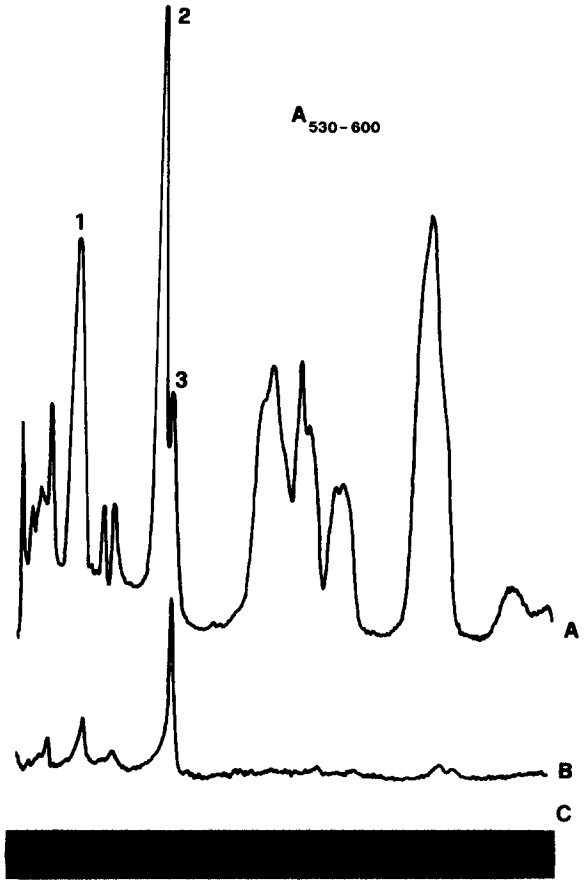

Fig.2. SDS-PAGE of cytochrome oxidase labeled with EMA. (A,B) Densitometric traces of the slab gel measured after electrophoresis. EMA absorbance was recorded at 530–600 nm. Each lane contained about 1 nmol enzyme. The numbers indicate the subunit sequence. In (A), cytochrome oxidase was dissolved in 3% SDS prior to its labeling with EMA (1:30 molar excess). In (B) the labeling was performed in 1% cholate as described in section 2. (C) Fluorography of (B) taken before the densitometric analysis.

Pretreatment of the enzyme with higher amounts of mersalyl (5:1 molar ratio) or with thionitrobenzoate-activated yeast, cytochrome *c* abolished completely the EMA labeling. This is an additional indication that subunit III was the labeled subunit by EMA.

Upon binding to the enzyme, EMA showed a typical spectral shift of the absorption maximum from 525 to 532 nm [14], and to 529 nm when the complex was denatured by SDS.

The stoichiometry of eosin binding to the native cytochrome oxidase in detergent solution was calculated from absorption spectra of the labeled enzyme (fig.3). It was found that the EMA/enzyme molar ratio was approx. 1. Such a finding confirms that only one cysteinyl residue of cytochrome oxidase subunit III was labeled under the above conditions. Comparison of the densitometric traces of subunit III labeled in SDS (fig.2A) and in cholate (fig.2B) showed an almost equivalent absorbance indicating that only one residue was reactive even in the partly denatured protein. Malatesta and Capaldi [6] demonstrated that cysteinyl 115 was the only modified cysteinyl residue in subunit III; Verheul et al. [7] found that even after dissociation in SDS only one cysteine was modified. From these results it is possible to conclude that EMA labeled cysteinyl residue 115.

Taking advantage of the possibility to monitor selectively subunit III, gel permeation experiments with intact and DCCD-modified enzymes were performed to detect a possible effect of the carbodiimide on the association of this subunit with the enzyme. The composition of the elution buffer [3] including 0.5 M NaSCN and 0.1% $C_{12}E_{10}$ was chosen to achieve a better resolution of subunit III from the whole complex. Fig.4 shows the elution profiles of the untreated (dotted line), and DCCD-treated enzyme (solid line) followed by the EMA

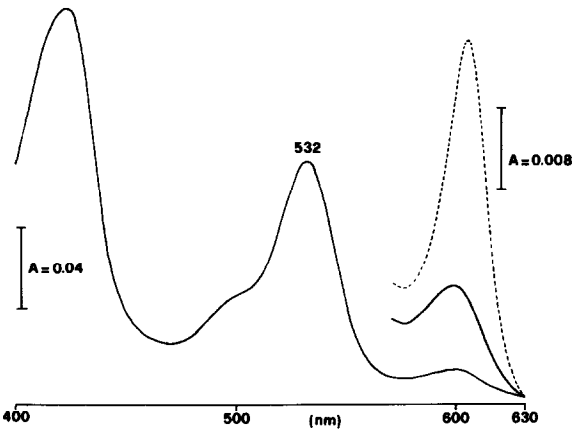

Fig.3. Absorption spectra of EMA-labeled cytochrome *c* oxidase. Solid line, air oxidized enzyme; dotted line, dithionite-reduced enzyme. The central peak is the absorption maximum of EMA (532 nm). The EMA/enzyme molar ratio was calculated to be about 1.

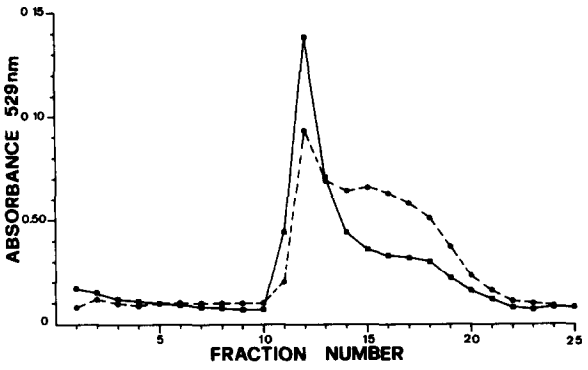

Fig.4. Gel filtration of EMA-labeled cytochrome *c* oxidase on Fractogel TSK HW-55(S). The column was equilibrated with 375 mM Tris-Cl, 75 mM glycine, 0.5 M NaSCN, 0.1% Triton X-100, 0.1% $C_{12}E_{10}$ (pH 9.5). The dotted line shows the elution profile of untreated enzyme, whereas the solid line shows that of DCCD-treated enzyme (DCCD 100:1 molar excess, 45 min at 20°C). The absorbance of eosin measured in the elution profiles, is an indicator of the presence of subunit III which was specifically labeled with EMA.

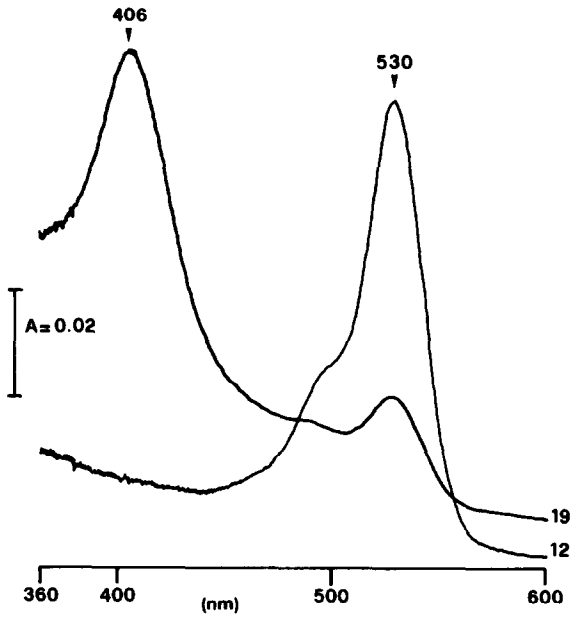

Fig.5. Absorption spectra of fractions 12 and 19 of fig.4, DCCD-treated enzyme. Fraction 12 exhibited only EMA absorption. Fraction 19 showed mainly heme absorption. The blue shift of heme absorption was due to the presence of NaSCN at pH 9.5.

absorbance. The peaks of fraction 12 contained mainly subunit III, in such an aggregated state that it was impossible to separate this material on SDS polyacrylamide gels. The use of larger amounts of SDS, urea, hexylsulfate and different types of organic solvents did not bring about the dissolution of the aggregated material which was found by SDS gel electrophoresis in the stacking gel and/or on the top of the running gel. Further evidence that DCCD treatment lowered the content of subunit III associated with the complex, could be obtained by the analysis of fraction 19, in which an equal heme absorbance was present, yet much less eosin absorbance than in the control (fig.5).

This observation may suggest that one of the possible effects of DCCD on the enzyme is that of loosening the interactions between subunit III and the rest of the complex.

REFERENCES

[1] Anderson, S., De Bruijn, M.H.L., Carlson, A.R., Eperon, I.C., Sanger, F. and Young, I.G. (1982) J. Mol. Biol. 156, 638.
[2] Casey, R.P., Thelen, M. and Azzi, A. (1980) J. Biol. Chem. 255, 3994–4000.
[3] Pentillä, T. (1983) Eur. J. Biochem. 133, 355–361.
[4] Thelen, M., O'Shea, P.S., Petrone, G. and Azzi, A. (1985) J. Biol. Chem. 260, in press.
[5] Prochaska, L.J., Bisson, R., Capaldi, R.A., Steffens, G.M.C. and Buse, G. (1981) Biochim. Biophys. Acta 637, 360–373.
[6] Malatesta, F. and Capaldi, R.A. (1982) Biochem. Biophys. Res. Commun. 109, 1180–1185.
[7] Verheul, F.E.A.M., Draijer, J.W., Muijers, A.O. and Van Gelder, B.F. (1982) Biochim. Biophys. Acta 681, 118–129.
[8] Nigg, E.A. and Cherry, R.J. (1979) Biochemistry 18, 3457–3465.
[9] Bürkli, A. and Cherry, R.J. (1981) Biochemistry 20, 138–145.
[10] Wagner, R. and Junge, W. (1982) Biochemistry 21, 1890–1899.
[11] Müller, M., Krebs, J.J.R., Cherry, R.J. and Kawato, S. (1982) J. Biol. Chem. 257, 1117–1120.
[12] Müller, M., Krebs, J.J.R., Cherry, R.J. and Kawato, S. (1984) J. Biol. Chem. 259, 3037–3043.
[13] Yu, C., Yu, L. and King, T.E. (1975) J. Biol. Chem. 250, 1383–1392.
[14] Cherry, R.J. (1978) Methods Enzymol. 54, 47–61.

[15] Kadenbach, B., Jarausch, J., Hartmann, R. and Merle, P. (1983) Anal. Biochem. 129, 517–521.
[16] Broger, C., Allemann, P. and Azzi, A. (1979) J. Appl. Biochem. 1, 455–459.
[17] Steck, G., Leuthard, P. and Bürk, R.R. (1980) Anal. Biochem. 107, 21–24.
[18] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 177, 265–275.
[19] Buse, G., Steffens, G.M.C. and Meinecke, L. (1983) in: Structure and Function of Membrane Proteins (Quagliariello, E. and Palmieri, F. eds) pp.131–138, Elsevier, Amsterdam, New York.