PHOTOAFFINITY LABELING OF YEAST CYTOCHROME OXIDASE WITH ARYLAZIDO CYTOCHROME *c* DERIVATIVES

Roberto BISSON and Heidi GUTWENIGER
Istituto di Patologia generale, Center for the study of Physiology of Mitochondria, University of Padova, Italy

and

Angelo AZZI
Medizinisch-chemisches Institut, University of Berne, Switzerland

Received 1 June 1978
Revised version received 22 June 1978

1. Introduction

The interaction of cytochrome *c* with cytochrome *c* oxidase, and the electron transfer occurring in the complex, are important events in the mitochondrial respiratory chain, leading to oxygen reduction, asymmetric proton release and conservation of the free energy of the redox reaction as an electrochemical gradient of hydrogen ions [1–3].

Among the approaches utilized to elucidate the structure of the cytochrome oxidase–cytochrome *c* complex, crosslinking of the components of the system has been recently employed by a number of investigators.

Using yeast cytochrome *c*, modified at its free sulfhydryl group by the reaction with 5,5′-dithiobis (2-nitrobenzoate), subunit III of yeast cytochrome *c* oxidase was indicated [4] to be close to the binding site for cytochrome *c*.

Using horse heart cytochrome *c* labeled with a 3-nitrophenylazido group at lysine 13, evidence was provided [5,6] that subunit II was the subunit nearest to cytochrome *c* in the complex with the beef-heart oxidase. This conclusion was also supported by [7] in which dithiobissuccininidylpropionate was used as crosslinking reagent. Using an azido-cytochrome *c* derivative, the binding site of cytochrome *c* was found [8] to occur in the two smallest subunits of cytochrome oxidase (9000 mol. wt and 11 000 mol. wt).

In this work yeast cytochrome oxidase was reacted with arylazido cytochrome *c* in order to compare the subunits of yeast and beef-heart cytochrome oxidase which are crosslinked when this derivative is used [5,6]. Furthermore, subunit II, which may migrate anomalously with respect to subunit III [9], was identified in our experimental conditions by labeling its highly reactive SH group with *N*-[^{14}C]ethylmaleimide.

2. Materials and methods

Preparations of cytochrome *c* oxidase [10] from beef-hearts, and of cytochrome *c* arylazido derivatives [5] were carried out according to published procedures. Cytochrome *c* oxidase from *Saccharomyces cerevisiae* was a very generous gift of Professor G. Schatz, Biocenter Basel, Polyacrylamide gel electrophoresis was carried out according to [11] and stained with Amido Black 10B. Labeling with *N*-[^{14}C]-ethylmaleimide (Amersham, England) was carried out according to [13]. Binding of cytochrome *c* derivatives to cytochrome oxidase was carried out as in [5,6]. Cytochrome *c* oxidase activity and concentration were measured according to [5]. The analysis of radioactivity was made after slicing the

gel and extracting it in Lipoluma–Lumasolve–H_2O in proportions of 9:1:0.1.

3. Results and discussion

Illumination of cytochrome *c* derivatives in the presence of cytochrome oxidase resulted in the formation of covalent complexes, which could not be dissociated by high ionic strength or by ion-exchange chromatography (cf. [5,6]).

In the presence of 5-fold excess of arylazido cytochrome *c* (table 1) illumination produced a covalent association in which cytochrome *c*:oxidase molar ratio exceeded the value of 1 or 2 expected for saturation of high and low affinity binding sites [13]. Such a result indicates that phospholipid sites are also involved in the binding of cytochrome *c*, and this is supported by the finding that only one polypeptide of cytochrome *c* oxidase is affected by arylazido cytochrome *c* binding.

The illumination of cytochrome oxidase–cytochrome *c* complexes produced a decrease in cytochrome oxidase enzymatic activity and this decrease was proportional to the disappearance of band II in the electrophoretic profile. This suggests that covalent association of cytochrome *c* with cytochrome oxidase results in a loss of enzymatic activity.

The polypeptide analysis of the arylazido cytochrome *c*–cytochrome oxidase complexes after illumination was carried out by polyacrylamide gel electrophoresis. The polypeptide patterns of either 22-arylazido- or 13,22-arylazido cytochrome *c* were only modified at the level of two bands in the densitometric profile shown in fig.1, namely band II

Table 1
Covalent binding of cytochrome *c* to yeast cytochrome oxidase

	Covalent binding (nmol cyt. *c*/ nmol cyt. *c* oxidase)	Activity (e^-/cyt.*c* oxidase/s)
Native	0.3	85
22-Arylazido-cyt. *c*	2.1	49
13,22-Arylazido-cyt. *c*	3.9	33

The samples were irradiated as in fig.1. They were subsequently purified by Amberlite CG-50 chromatography to remove unreacted cytochrome *c* [6]. Oxidase activity was measured spectrophotometrically in 40 mM P_i buffer, pH 7.4, 0.5% Tween-80, 0.2 mM EDTA and 32 μM cytochrome *c*

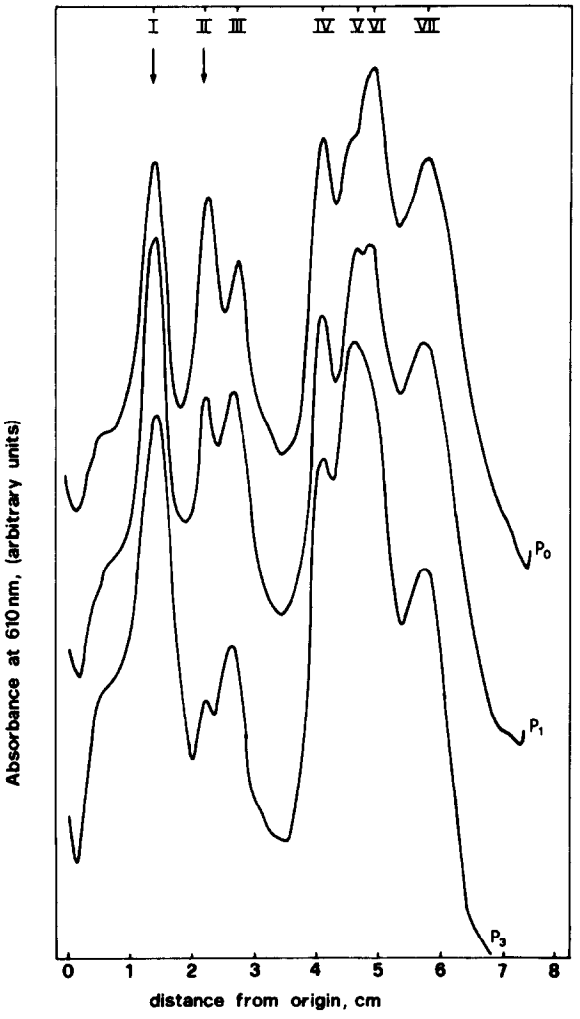

Fig.1. Polyacrylamide gel electrophoresis of yeast cytochrome oxidase after illumination in the presence of arylazido cytochrome *c* derivatives. The incubation medium was the following: 2 mM Tris-acetate, pH 7.0, 100 μM EDTA, 0.01% Tween-80, 2.2 μM yeast cytochrome oxidase and 12 μM cytochrome *c* (P_0) or arylazido cytochrome *c* derivative at lysine 22 (P_1) and lysines 22 plus 13 (P_3). Illumination of the samples was obtained with a 100 W ultraviolet lamp at 1 cm distance from the samples for 30 min, through a glass filter. Polyacrylamide gel electrophoresis was carried out in 15% acrylamide gels with a step of 2.5% gel under the conditions in [6]. The buffer employed was 150 mM Tris–acetate, pH 8.2, 1.5% sodium dodecylsulfate.

and I. With respect to a control experiment (oxidase illuminated in the presence of native cytochrome *c*) cytochrome *c* derivatives produced a marked decrease in band II and an increase in I. The two mentioned changes were always much more pronounced with the 13,22-arylazido cytochrome *c* than with the 22-derivates, however, in beef-heart oxidase [5,6], the polypeptide pattern was not at all modified by the 22-derivative. This suggests that a larger area of cytochrome *c* may be in contact with the yeast enzyme.

The two phenomena were absolutely dependent on the presence of azido-cytochrome *c* and on its concentration (fig.2). The plot indicates that increasing the concentration of 13,22-arylazido-cytochrome *c* produces a progressive diminution of the amplitude of band II. On extrapolation of the data points obtained at low cytochrome *c* concentration to zero ordinate an intercept can be obtained at ~ 1 mol cyt. *c*/oxidase.

The finding of a specific diminution of band II (mol. wt 33 000) and increase of band I (mol. wt 40 000) when cytochrome *c* is covalently bound to the oxidase, indicates that a crosslinked polypeptide has been formed between the protein of band II and

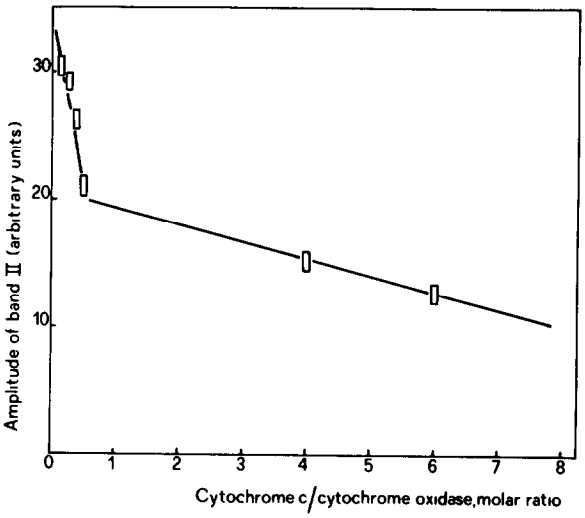

Fig.2. Decrease in band II of yeast cytochrome oxidase as a function of the concentration of arylazido cytochrome *c*. Experimental conditions were as in fig.1 except that the concentration of cytochrome *c* (13,22 arylazido derivative) was varied to obtain the plotted ratios.

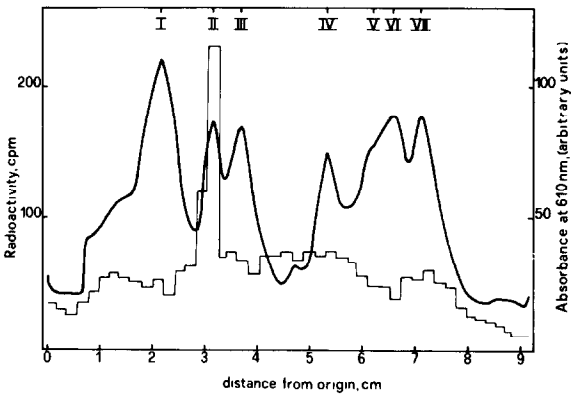

Fig.3. Labeling of yeast cytochrome *c* oxidase by *N*-[^{14}C]-ethylmaleimide. The reaction was carried out in the following medium: 10 mM Tris–HCl, pH 7.0, 10 mM EDTA, 100 mM KCl, 10 mM NaF, 0.5% Tween-20 and 200 μM *N*-[^{14}C]-ethylmaleimide (spec. act. 5 mCi/mmol). After 15 min incubation at room temperature the reaction was ended by the addition of 2 mM β-mercaptoethanol. The sample was desalted in Sephadex G-25 using 0.5 mM EDTA–Tris, pH 7.0, and 0.01% Tween-20 as eluting buffer and lyophilized samples (200 μg protein) were applied to the slab gel in fig.1. After the electrophoretic run the gel was devided in two parts, one of which was stained; the other was cut in 1 mm slices. Radioactivity was analyzed in Lipoluma–Lumasolve–H_2O, 9:1:0.1.

cytochrome *c*, and that this peptide has a molecular weight close to that of band I.

In order to compare the electrophoretic migration of subunits II and III in our system with that found [4], yeast cytochrome oxidase was reacted with *N*-[^{14}C]ethylmaleimide (fig.3), which under our experimental conditions uniquely labeled subunit II, in analogy with [4]. Thus, the subunit labeled by azido cytochrome *c* in yeast cytochrome oxidase, was the subunit bearing the very reactive SH-group, i.e., subunit II. It appears therefore that the interaction between cytochrome *c* and cytochrome oxidase is similar in the yeast and beef-heart enzymes.

4. Conclusions

The use of a photolabeling technique has shown that polypeptide II (mol. wt 33 000) of yeast cytochrome *c* oxidase is the only one which covalently binds arylazido cytochrome *c* derivatives. Thus, the

only difference between yeast and beef-heart cytochrome *c* oxidase, with respect to the interaction with cytochrome *c*, is in the region of cytochrome *c* which is in contact with polypeptide II. Whereas, for the yeast enzyme, both lysine 22 and lysine 13 are involved in binding, with the beef-heart enzyme only the involvement of lysine 13 can be detected.

This result is in agreement with the mapping of the binding area of cytochrome *c* on beef cytochrome oxidase [13]. Our findings, indicating that subunit II is involved in the binding site(s) of cytochrome *c*, are at variance with the results in [4], where iso-cytochrome *c*, modified at cysteine 107, binds to subunit III. The results [8], indicating that subunit VI and VII form the binding site of cytochrome *c*, may be due to the use of a long crosslinking agent.

The scheme of fig.4 attempts to combine all the present information on the interaction of cytochrome *c* with neighbouring subunits, including the distance data [15,16], from energy transfer experiments. Such a diagram, although somewhat speculative, is a useful model for the future investigation and is the basis of our present studies, using a variety of photoaffinity labels.

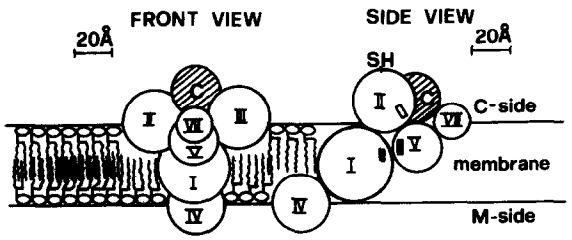

Fig.4. Schematic diagrams of the interaction of cytochrome *c* with cytochrome oxidase.

Acknowledgements

This work was partly supported by Swiss National Fond grant no. 3.228-0.77, Emil Barell-Stiftung and Clark Joller-Fond. The valuable discussions with Dr Cesare Montecucco and the technical assistance of Mr Mario Santato are gratefully acknowledged.

References

[1] Malmström, B. G. (1973) Quart. Rev. Biophys. 6, 389–429.
[2] Caughey, W. S., Wallace, W. J., Volpe, J. A. and Yoshikawa, S. (1976) in: The Enzymes (Boyer, P. D. ed) pp. 299–344, Academic Press, NY.
[3] Wikström, M. K. F. and Saari, H. T. (1977) Biochim. Biophys. Acta 462, 347–361.
[4] Birchmeier, W., Kohler, C. E. and Schatz, G. (1976) Proc. Natl. Acad. Sci. USA 70, 3245–3249.
[5] Bisson, R., Gutweniger, H., Montecucco, C., Colonna, R., Zanotti, A. and Azzi, A. (1977) FEBS Lett. 81, 147–150.
[6] Bisson, R., Azzi, A., Gutweniger, H., Colonna, R., Montecucco, C. and Zanotti, A. (1978) J. Biol. Chem. 253, 1874–1880.
[7] Briggs, M. M. and Capaldi, R. A. (1978) Biochem. Biophys. Res. Commun. 80, 553–559.
[8] Ereciñska, M. (1977) Biochem. Biophys. Res. Commun. 76, 495–501.
[9] Capaldi, R. A., Bell, R. L. and Branghek, T. (1977) Biochem. Biophys. Res. Commun. 74, 425–433.
[10] Yu, C. A., Yu, L. and King, T. E. (1975) J. Biol. Chem. 250, 1383–1392.
[11] Yamamoto, T. and Orii, Y. (1974) J. Biochem. (Tokyo) 75, 1081–1089.
[12] Kornblatt, J. A., Chen, W. L., Hsia, J. C. and Williams, G. R. (1975) Can. J. Biochem. 53, 364–370.
[13] Miller, S. F., Brautigan, D. L. and Margoliash, E. (1978) J. Biol. Chem. 253, 149–159.
[14] Poyton, R. D. and Schatz, G. (1975) J. Biol. Chem. 250, 752–761.
[15] Vanderkooi, J. M., Landesberg, R., Hayden, G. W. and Owen, C. S. (1977) Eur. J. Biochem 81, 339–347.
[16] Dockter, M. E., Steinemann, A. and Schatz, G. (1978) J. Biol. Chem. 253, 311–317.