COVALENT BINDING OF ARYLAZIDO DERIVATIVES OF CYTOCHROME *c* TO CYTOCHROME OXIDASE

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1. Introduction

The interaction among proteins in biological membranes is an important phenomenon responsible for a number of membrane catalyzed reactions. Cross-linking reagents and spectroscopic techniques have been employed to study nearest neighbor interactions and distances between proteins in membranes [1–3].

Cross-linking of proteins, while largely employed in membrane studies, suffers from the disadvantage that the reactive groups of the reagents select the target groups of the proteins on the basis of their chemical nature and reactivity [2]. Consequently to this, cross-linking of membrane proteins is a function of two parameters, i.e. vicinity and availability of specific chemical groups.

In contrast photoactivation of arylazido derivatives yields nitrenes of very broad specificity [4,5]. This in turn allows one to circumvent the problem of specific group availability at the surface of neighbouring proteins and to define their interactions with less uncertainty.

The proteins chosen in this study were horse-heart cytochrome *c*, and beef-heart cytochrome oxidase. The data suggest that cytochrome *c* labeled at lysyl 13 residue with a 4-nitrophenylazide group forms a covalent complex with the polypeptide of mol. wt 23 700 (band II) of cytochrome oxidase. When the label is located at lysyl 22 residue, no covalent protein binding is observed.

2. Materials and methods

Cytochrome oxidase was prepared according to the method of Yu et al. [6], and had a heme content of 11.3 nmol/mg protein, a phospholipid content of 10% (w/w) and an activity of 95 nmol cytochrome $c.s^{-1}$. nmol cytochrome $oxidase^{-1}$.

4-Fluoro-3-nitrophenylazide was prepared according to Fleet et al. [7] from 4-fluoro-3-nitro-1-aminobenzene and crystallized from light petroleum (m.p. 52°C). Electrophoresis in polyacrylamide gel in the presence of Na-dodecylsulfate was carried out according to Yamamoto and Orii [8]. Cellulose acetate electrophoresis (Cellogel, Labometrics, Milano) was carried out in Tris–acetate buffer (pH 7.2, 50 mM). Protein staining was obtained with Amido Black 10B. Binding of cytochrome *c* derivatives to the oxidase was carried out under the following conditions: 2 μM cytochrome oxidase, 0.5 mM EDTA–Tris, pH 6.5, 0.1% Tween 80, 12 μM cytochrome *c* (or arylazido-derivatives). Illumination was obtained through a 100 W ultraviolet lamp, shielded with a glass filter, for 45 min at 0°C under stirring, at a distance of 10 mm. Separation of non-covalently-bound cytochrome *c* was carried out in small Amberlite CG-50 columns (1 × 80 mM) equilibrated and eluted with 50 mM ammonium acetate. The eluate was lyophilized and dissolved in a small volume for spectral analyses and electrophoresis. Spectrophotometric measurements were carried out in an Hitachi spectrophotometer (Model 124) and

protein was determined according to Lowry et al. [9]. All reagents employed were commercially available reagent grade products.

3. Results and discussion

Incubation of horse heart cytochrome *c* (400 mg) with 4-fluoro-3-nitrophenylazide in 6 ml 0.1 M bicarbonate buffer containing 7% ethanol, pH 9.5 at 37°C, for 60 h, followed by Sephadex G-25 filtration and Amberlite CG-50 chromatography (1 X 52 cm column equilibrated with 50 mM ammonium acetate and eluted with 200 mM ammonium acetate, pH 7.2) resulted in the separation of four products. The first derivative eluted was highly labeled protein mixture, the second was a doubly-labeled derivative, the third (named P_2) and the fourth (named P_1) were mono-labeled derivatives and the last elution product was unlabeled cytochrome *c*. The extent of labeling was calculated according to Wada and Okunuki [10] from the electrophoretic mobility of the derivatives and from the absorbance of the arylazido derivatives at 480 nm [4] (table 1).

In order to establish the position of the label introduced, the heme undecapeptides of the different derivatives were prepared according to Harbury and Loach [11] and analyzed in TLC (silica gel; eluent butanol/acetic acid/pyridine/H_2O 10:15:3:12). Native and fraction P_1 heme undecapeptide had exactly the same velocity of migration, while the peptide formed from fraction P_2 was slightly faster, indicating that this peptide was modified with respect to that from native cytochrome *c* and P_1. The only residue modified in the heme undecapeptide by the reagent employed could be lysyl 13, the only lysyl residue of this peptide.

Table 1
Recovery of arylazido cytochrome *c* derivatives from Amberlite CG-50 column

Fraction	Recovery (%)	No. labeled residues
P_0	27	None
P_1	25	1
P_2	27	1
P_3	24	2
P_4	7	0

Further evidence for this indication was obtained after trypsin digestion of the heme undecapeptides. Native and P_1 undecapeptides were digested, while that from fraction P_2 was not, confirming the presence of the label at the ε-amino group of lysyl 13 in this derivative. From the notion that the two first lysyl residues labeled in cytochrome *c* by amino-group reagents are lysyl 13 and 22, it can be inferred that the fraction P_1 not being labeled at residue 13 was most probably labeled at residue 22 [10].

The catalytic activity of the derivative of cytochrome *c* with the label at position 22 was essentially maintained, both in terms of V_{max} and K_m, when assayed in the presence of cytochrome oxidase (cf. ref. [12]). The derivative labeled at position 13 had a K_m four-fold higher than native cytochrome *c*, consistent with the data indicating in lysine 13 an essential residue for the interaction with the oxidase [10,13]. Illumination by ultraviolet light of cytochrome oxidase in the presence of a six-fold molar excess of cytochrome *c* derivative P_1 resulted in the formation of a complex which could not be dissociated by ammonium sulfate precipitation or by passage through Amberlite CG-50. Such a complex (0.1 cytochrome *c*/oxidase) appeared

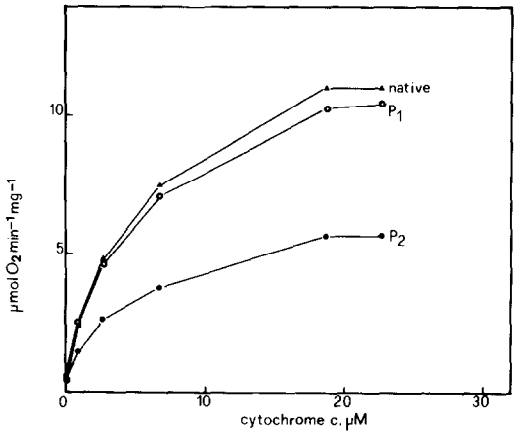

Fig.1. Catalytic activity of cytochrome *c*–cytochrome oxidase complexes. The catalytic activity of the complexes between cytochrome oxidase and cytochrome *c* derivatives (or native cytochrome *c*) was measured in a polarographic apparatus using a Clark type oxygen electrode, in the following assay system: 50 mM K-phosphate, pH 6.0, 1 mM EDTA, 20 mM Na-ascorbate, 0.7 mM tetramethyl-*p*-phenylene-diamine, 0.1 μM cytochrome oxidase (or its complexes), 0.6 mg asolectin in total vol. 3.5 ml.

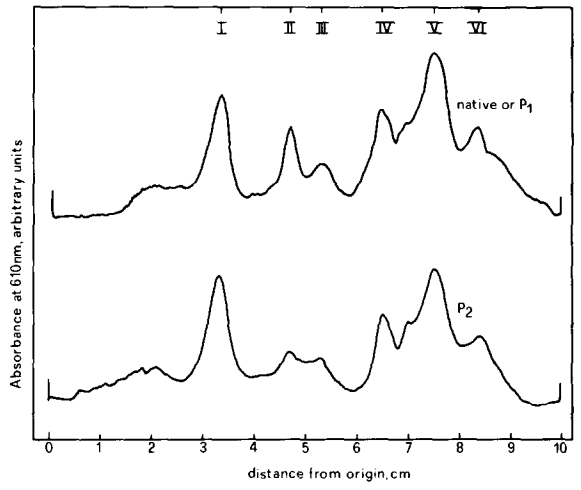

Fig.2. Electrophoretic analysis of polypeptide composition of the complexes formed between cytochrome oxidase and arylazido cytochrome *c* derivatives. Experimental details are described in 'Materials and methods'. In order to increase labeling, after illumination the mixture of cytochrome *c* and oxidase was passed through a small Amberlite CG-50 column to eliminate free cytochrome *c*. Cytochrome *c* 12 μM, (or arylazido cytochrome *c*) were subsequently added and a second 45 min illumination period was initiated. The samples were purified again in the small Amberlite column, lyophilized and analyzed. The Raman numerals indicate the positions of the cytochrome oxidase subunits.

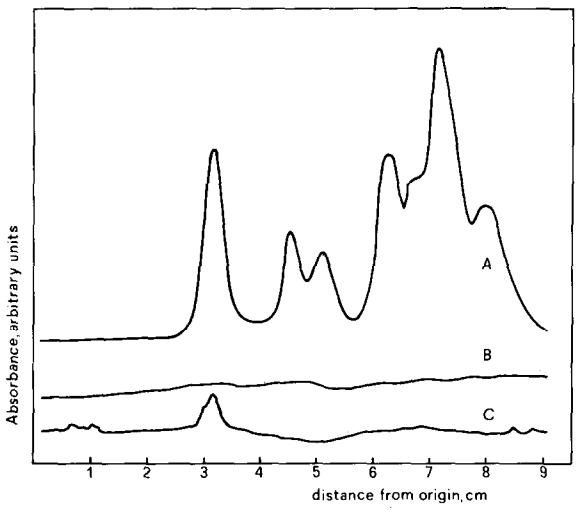

Fig.3. Location of crosslinked cytochrome *c* polypeptide in unstained polyacrylamide gels. The experimental conditions were those of fig.2. The gel was not colored and scanned at 410 nm. Trace A is a polypeptide analysis of native cytochrome oxidase, stained with amido black and scanned at 610 nm. Trace B is the electrophoresis densitometric trace of cytochrome oxidase–P_1 complex and trace C that of cytochrome oxidase–P_2 complex analyzed at 410 nm.

thus to be formed through a covalent bond between the oxidase and cytochrome *c*. The complex formed between P_2 and the oxidase (0.8 cytochrome *c*/oxidase) had the same characteristics. The activity of the two complexes in the presence of different concentrations of native cytochrome *c* is reported in fig.1. In the absence of added native cytochrome *c*, covalently bound cytochrome *c* was not able to function as an electron carrier. In the presence of excess native cytochrome *c* the oxidase–P_1 complex had the same activity (within experimental error) of native cytochrome *c* while the oxidase–P_2 complex was inhibited by more than 50%. Thus when the label was located at position 13, larger covalent binding and inhibition of catalytic activity of the oxidase occurred.

The polypeptide profile of the two complexes, as obtained in polyacrylamide gel electrophoresis in the presence of Na-dodecylsulfate is shown in fig.2. No difference in the number, relative mobility, and dimensions of the different bands of the oxidase could be detected after illumination of the enzyme in the presence of fraction P_1. Instead, a large diminution of band II was evident in the complex obtained after illumination of the oxidase P_2. Such a diminution was associated with an increase in band I (25%) relative to the total area of the profile. Also, the presence in unstained gels, after scanning at 410 nm, of a band corresponding to band I of the stained gel could be detected. Such a band was not present either with native or fraction P_1 cytochrome *c* derivative (fig.3).

The diminution of band II, the increase in band I and the presence of a heme *c* band corresponding to band I indicate that cytochrome *c* derivative labeled at lysine 13 has become covalently attached to band II. In fact the mol. wt of band II is 23 700, that of cytochrome *c* 12 400 and that of the summation polypeptide formed from them 36 100. Such a summation polypeptide would coincide in terms of electrophoretic mobility with band I polypeptide of mol. wt 35 000.

The labeling of the polypeptide migrating as band II by cytochrome *c* appears a specific event, under the

experimental conditions employed in this study. If the binding were the consequence of the easier interaction of cytochrome *c* with the more exposed subunits, a labeling of subunit V, VI and III would be also expected. Also the labeling of a single subunit, despite the excess of cytochrome *c* with respect to the oxidase, points to a selected interaction between subunit II and cytochrome *c*. Finally, the inability of the derivative labeled in position 22 to establish covalent binding with the oxidase protein is further support to the idea that subunit II interacts with cytochrome *c* not only specifically but also with a restricted portion of its surface. The recent data of Birchmeier et al. [14] indicating that subunit III of yeast cytochrome *c* oxidase is covalently labeled by a dithionitro benzoate iso-1-cytochrome *c* (with the label at position 107) are difficult to compare with the present study, due to the lack of knowledge concerning the homology between yeast and beef-heart cytochrome oxidase polypeptides. Similarly the apparent discrepancy between the conclusions of this study and that of Erecinska et al. [15] may be the consequence of the differences in the systems employed. The formation of an active covalent complex between an arylazido cytochrome *c* derivative and cytochrome oxidase was achieved, in the study of Erecinska et al. [15] in intact mitochondria, while in this study purified cytochrome oxidase was employed.

The polypeptide of beef-heart cytochrome oxidase having mol. wt 23 700 may represent therefore either the natural binding site of cytochrome *c* or may be close to it.

Acknowledgements

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