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INTERACTION OF REDUCED AND OXIDIZED CYTOCHROME c WITH THE MITOCHONDRIAL CYTOCHROME c OXIDASE AND bc_1-COMPLEX

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Making use of a hetero-bifunctional reagent (succinimidyl 4-(p-maleimidophenyl)butyrate, SMPB), yeast cytochrome c was linked through a thioether bond to the maleimide group whereas the active N-hydroxysuccinimide ester site of the SMPB was used for the reaction with the primary amino groups of Affi-gel 102. The capacity and stability (also to reducing agents) of the column were greatly improved relative to previous systems. This new gel allowed the study of the interactions of cytochrome c oxidase and reductase with reduced and oxidized cytochrome c. For cytochrome c oxidase a significant difference in the interaction with ferri- and ferro-cytochrome c was observed but no such a difference was seen in the case of cytochrome c reductase.

Cytochrome c, the physiological electron carrier between complex III (bc_1-complex) and complex IV (cytochrome c oxidase) is one of the best functionally and structurally characterized proteins. X-ray diffraction (1,2) together with ^1H NMR studies, have shown significant differences in the conformation of the reduced and oxidized state (3, 4). The control of redox properties of cytochrome c by electrostatic interactions have been discussed recently (5) in terms of a redox dependent conformational change mostly affecting the base of the heme around the propionate groups (6,7). Studies on the interaction of cytochrome c with other proteins (8-14) have shown that the binding domain for both cytochrome c oxidase and reductase, is located in the front part of the molecule, containing the top of the exposed heme edge.

Chemical crosslinking experiments have shown that cytochrome c_1 (15) and subunit II (16) are responsible for the interaction with cytochrome c in

Abbreviation: SMPB, Succinimidyl 4- (p-maleimidophenyl) butyrate

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complex III and in complex IV respectively, probably through clusters of negatively charged residues (17,18).

In this study we have investigated the possibility that a conformational difference between ferri- and ferro-cytochrome c (and possibly similar differences in the reduced and oxidised hemoprotein with which it forms a complex) results in a change in the affinity for the isolated cytochrome reductase and oxidase. This work was carried out in an attempt to confirm previous indications of a difference in affinity between the reduced and oxidized cytochrome c for cytochrome c oxidase (19)

MATERIALS AND METHODS

Beef heart cytochrome c oxidase was prepared according to a standard procedure (20). Cytochrome bc_1-complex was a generous gift of Dr. M. Nalecz. Spectral analysis were carried out with an Aminco-DW 2a spectrophotometer. Affi-gel 102 was obtained from Bio-Rad, SMPB from Pierce, cytochrome c type VII from Sigma and Triton X-100 from Fluka. The other substances were of the purest grade commercially available.

Preparation of the Affi-gel 102 - SMPB

To 15 ml of preswollen Affi-gel 102 washed three times with 25 mM Na-phosphate buffer, pH 7.4, 10 ml of anhydrous dioxane was added. After stirring and centrifugation (3x) the gel was suspended in 5 ml of dioxane.

20 mg of SMPB were dissolved in 2 ml of dioxane and then added to 15 ml of affi-gel 102, carefully stirred under nitrogen for 3 hours at room temperature in the dark. Unbound SMPB was washed away with dioxane (2x) and with 10 ml of 25 mM Na-phosphate buffer pH 7.4 (8x).

Preparation of the Affi-gel 102 - SMPB - yeast cytochrome c (Fig. 1)

Cytochrome c was reduced by addition of $Na_2S_2O_4$ and passed through a Sephadex G-25 column. To the Affi-gel 102-SMPB as prepared and described above (suspended in 10 ml of 25 mM Na-phosphate buffer, pH 7.4) reduced cytochrome c (927 nmoles) was added. This mixture was carefully stirred for 12 hours at 4 °C in the dark. Three washing cycles were used to remove the unbound cytochrome c (each with 1 M NaCl, 25 mM Na-phosphate, pH 7.4, the same plus $Na_2S_2O_4$, pH 7.4 and finally with 1 M NaCl, 25 mM Na-phosphate, pH 7.4). More than 80% of the added cytochrome c was bound to the resin (739 nmoles). The gel was stored in 1 M NaCl, 25 mM Na-phosphate, 0.02% Na-azide, pH 7.4.

Affinity chromatography of complex IV and III under oxidizing and reducing conditions

5 ml of the gel was poured into a column (1x10 cm), washed with 50 ml of 9 mM Tris-HCl, 1 mM EDTA, 1 mM ferricyanide, pH 7.4 to oxidize fully cytochrome c. The column was washed with 50 ml of 9 mM Tris-HCl, 1mM EDTA, pH 7.4 and equilibrated with the same buffer containing 0.05% Triton X-100. 10 nmol cytochrome c oxidase in 8 ml of the same buffer was applied to the column at a flow rate of 10 ml/h. After loading, the column was washed until no heme was detected. A linear gradient between 0 and 100 mM NaCl in the washing buffer was applied at the same flow rate (total 40 ml). Fractions of 1.6 ml were collected and analyzed spectrophotometrically using an Aminco DW-2a spectrophotometer.

The experiment using reducing conditions was carried out as above with the exception that the cytochrome c of the gel was first fully reduced with $Na_2S_2O_4$ and the washing- and elution buffer contained 7 mM Tris-HCl,

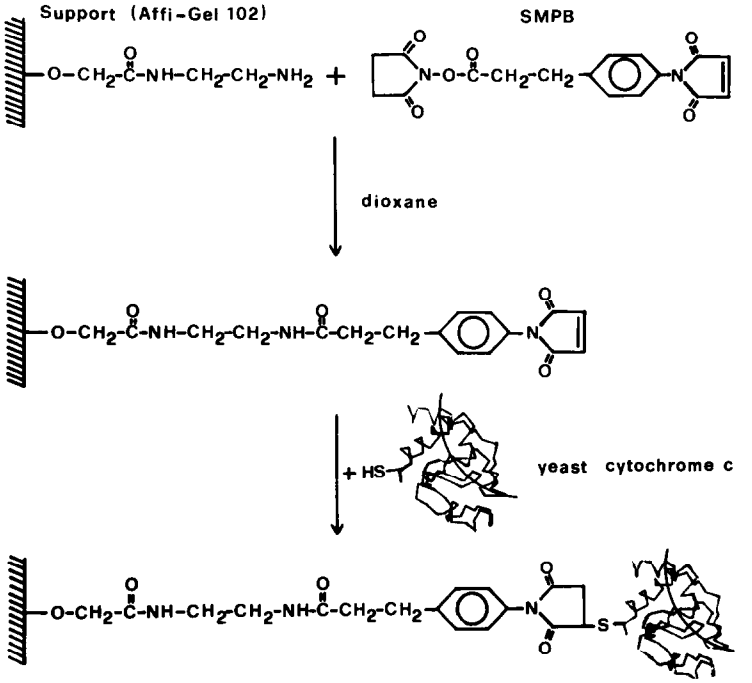

Fig.1. Scheme of the reactions involved in the preparation of the cytochrome c gel using the hetero-bifunctional SMPB reagent for joining the ligand (yeast cytochrome c) through a thioether bond by its maleimide function and to the solid matrix (Affi-gel 102) by the N-hydroxy-succinimide active ester function.

2 mM $Na_2S_2O_4$, 1 mM EDTA, 0.05% Triton X-100, pH 7.4. The experiments with the bc_1-complex were carried out under identical conditions as described for the cytochrome c oxidase. For the experiments under oxidizing and reducing conditions 8 nmoles of the bc_1-complex were used.
The concentrations of cytochromes aa_3, b, c and c_1 were calculated using the extinction coefficients of 13.4 (605-630nm), 25.6 (562-577nm), 19.6 (550-540nm) and 20.1 $mM^{-1}cm^{-1}$ (553-540 nm), respectively. The protein concentration was estimated using the method of Wang and Smith (21) or Spector (22).

RESULTS AND DISCUSSION

The interaction between cytochrome c and other redox proteins can be realised using an affinity column provided that cytochrome c is attached to it by a stable covalent bond, that the binding site is free for the interaction with cytochrome c oxidase and bc_1-complex and that its bond with the matrix is stable under reducing conditions. The previously described system (18, 23-27) containing a disulfide bridge through which cytochrome c was attached to a Sepharose gel, may not be used under strongly reducing conditions. This prompted us to construct a new type of cytochrome c affinity column making use of a hetero-bifunctional reagent

(SMPB) which, through its N-hydroxysuccinimide active ester was attached to the amino groups of Affi-gel 102 and through its maleimide group to yeast cytochrome c, forming a thioether bond with the SH-group of the cysteine residue near the C-terminus. Since the two functional groups of the hetero-bifunctional reagent have distinctly different reactivities it was possible to attach cytochrome c efficiently and without auto-crosslinking. To 1 ml of gel 50 n moles of yeast cytochrome c could be attached, to which 11 nmoles of cytochrome c oxidase per ml of gel. One out of five bound cytochrome c molecules resulted thus to be available for the interaction with cytochrome c oxidase. The stable thioether bond through which the cytochrome c was attached to the support permitted work to be carried out under strongly reducing conditions.

The elution pattern of pure cytochrome c oxidase using this new cytochrome c gel under oxidizing and reducing conditions is shown in Fig.2. Under oxidizing conditions the peak of the cytochrome c oxidase was found in fraction 11 (36 mM NaCl). Under reducing conditions, the maximal heme aa_3 concentration was found in fraction 14 (47 mM NaCl). This is interpreted in the sense that a difference in the affinity of

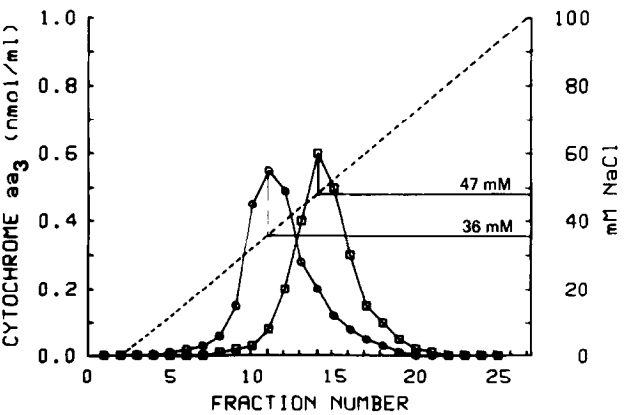

Fig.2. Elution pattern of cytochrome c oxidase. Elution was obtained with 9 mM Tris-HCl, 1 mM EDTA, 0.05% Triton X-100, pH 7.4, under oxidizing conditions; 7 mM Tris-HCl, 2 mM $Na_2S_2O_4$, 1 mM EDTA, 0.05% Triton X-100, pH 7.4 under reducing conditions, both with a linear 0-100 mM NaCl gradient. O cytochrome aa_3 under oxidizing conditions, □ cytochrome aa_3 under reducing conditions.

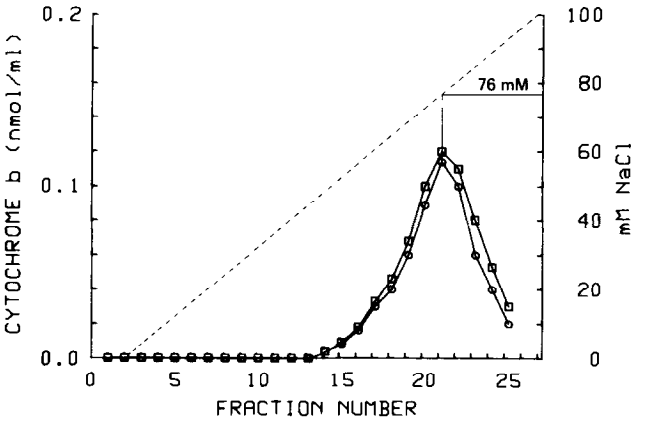

Fig.3. Elution pattern of bc_1-complex. Elution was obtained with the same buffers as described for cytochrome aa_3. O cytochrome b under oxidizing conditions. □cytochrome b under reducing conditions.

cytochrome c oxidase for ferri- and ferro-cytochrome c may be expressed as a NaCl salt concentration difference of 11 mM.

Similar experiments were carried out under identical conditions using isolated bc_1-complex. As it can be seen in Fig.3 no difference was found in the salt concentration detaching the bound enzyme. For ferri- as well as for ferrro-cytochrome c the maximal heme b concentration was found to be in fraction 21 corresponding to 76 mM NaCl.

The assumption that subunit II of the cytochrome c oxidase (which is the binding site for the interaction with cytochrome c) contains at least one of the two hemes (a and a_3, respectively) is rather well justified (28-30). Under the reducing conditions applied ($Na_2S_2O_4$) the hemes a and a_3 become and remain fully reduced. The reduced state of the hemes may be responsible for a small overall conformational change in the cytochrome c oxidase complex (31,32), and most probably this conformational change is also expressed in subunit II, which contains at least one copper and binds cytochrome c (17,19). As a consequence of this, a better fit between cytochrome c and oxidase binding may be expected, with the consequence of a stronger interaction. The complex of cytochrome c oxidase-cytochrome c is mainly ionic in nature, as indicated by its salt dissociation effect. The higher salt concentration needed for its

dissociation in the reduced form may indicate that more or stronger salt bridges are present at the interaction domains in the reduced system. In the case of the bc_1-complex where the cytochrome c_1 is the binding partner for cytochrome c (16), a conformational change might be much less expressed because there the heme is covalently attached to the protein, giving rise to a more rigid structure. It is likely that the bc_1-complex has always an optimal conformation for the interaction with the cytochrome c which does not depend upon its redox state.

The findings presented above support a model suggested by Capaldi et al. (33) in which reduced cytochrome c is detached from its sites on the bc_1-complex after reduction followed by binding to cytochrome c oxidase. Reduced cytochrome c, once having donated its electron to cytochrome c oxidase, becomes oxidized and in this state there is a greater probability to detach from it. Binding to the bc_1-complex will be now favoured by the higher affinity for it than for the oxidase.

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