CYTOCHROME bc_1 AND CYTOCHROME OXIDASE CAN BIND TO THE SAME SURFACE DOMAIN OF THE CYTOCHROME *c* MOLECULE

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1. Introduction

Two contrasting mechanisms can be envisaged for the cytochrome *c* catalyzed electron transfer from cytochrome c_1 (a component of the cytochrome bc_1 complex or ubiquinone cytochrome *c* reductase) to cytochrome oxidase. In one, called the 'static' mechanism, cytochrome *c* is bound simultaneously to cytochrome bc_1 and to cytochrome oxidase accepting electrons from cytochrome c_1 and donating electrons to the oxidase. It is unknown whether such a ternary complex exists in the inner mitochondrial membrane but it does form in vitro [1,2]. In the alternative, 'dynamic' mechanism, cytochrome *c* oscillates between bc_1 and oxidase carrying electrons hence and forth. The movement would be over a short distance in the plane of the inner membrane. Two spatially separated binding sites for bc_1 and oxidase on the cytochrome *c* molecule are predicted by the 'static' mechanism whereas identical or overlapping binding sites would conform to the 'dynamic' mechanism.

A binding site for the oxidase on the cytochrome *c* molecule was proposed [3]. The proposal was corroborated by the comparison of the chemical reactivity of lysine side chains in free and in oxidase-bound cytochrome *c*.

A binding site very similar to the one proposed in [3] was deduced from the oxidation kinetics of several singly substituted cytochrome *c* derivatives [4,5]. Here we report a binding site for cytochrome bc_1 on cytochrome *c* which is very similar or even identical to the binding site for the oxidase.

2. Materials and methods

The cytochrome bc_1 complex (4.4 nmol c_1/mg based on $\Delta\varepsilon^{red}_{552.5-540} = 17.5\ mM^{-1}$) was isolated from beef heart according to [6]. This complex is prepared in the presence of antimycin A and carboxyatractyloside (both from Boehringer) and does not catalyze electron transfer from hydroquinone to ferricytochrome *c* [6]. Active bc_1 complex (spec. act. 10.7 μmol ferricytochrome *c* reduced.min^{-1}.mg^{-1}, 3.9 nmol c_1/mg) was prepared according to [7]. All other materials were as in [3].

Differential reductive methylation and acetylation of the lysine residues of cytochrome *c* (horse heart) was performed as in [3]. Briefly, ferricytochrome *c* (50 μM), in the presence or absence of cytochrome bc_1 (50 μM in c_1) in 20 mM triethanolamine·HCl, 0.2% Tween-80, pH 8.2, was treated with a small amount of ^{3}H-labeled reagent. In some experiments ^{3}H-labeling of free cytochrome *c* was done with cytochrome bc_1 present but adding 0.25 M NaCl to the buffer. The complex does not form at high ionic strength [2,8]. Formaldehyde (750 μM) plus KB^3H_4 (200 μM) or [^{3}H]acetic anhydride (~250 μM) were used for reductive methylation [9] and acetylation, respectively. The ^{3}H-labeled cytochrome *c* derivatives contained less than one [^{3}H]methyl or [^{3}H]acetyl group per molecule on average. Equimolar amounts of the two derivatives which had been ^{3}H-labeled in the free form and bound to bc_1, respectively, were each mixed with equimolar amounts of uniformly [^{14}C]methylated or [^{14}C]acetylated cytochrome *c* derivatives. The mixtures were treated with non-

labeled reagents in excess. The chemically homogeneous but isotopically heterogeneous derivatives were digested by thermolysin or pepsin. Labeled peptides were separated and isolated by paper chromatography and high-voltage paper electrophoresis ('fingerprints') and analyzed for amino acid composition and $^{3}H/^{14}C$ radioactivity content. The $^{3}H/^{14}C$ ratio of a lysine residue (or residues) labeled in free cytochrome *c* was divided by the $^{3}H/^{14}C$ ratio of the same residue(s) labeled in bc_1-bound cytochrome *c*. The number obtained is called the shielding factor *R*. Thus stronger shielding of the lysine residues in the bc_1-bound cytochrome *c* leads to higher *R*-values.

3. Results

Cytochrome *c* forms a 1:1 complex with isolated cytochrome c_1 or bc_1 [8,1,2]. We have confirmed the existence of the complex with active [7] and inactive [6] cytochrome bc_1. [^{3}H]Methylated cytochrome *c*, 10 nmol [3] and 10 nmol (with respect to c_1) cytochrome bc_1 in 200 μl were chromatographed on a column of Sephadex G-200 in triethanolamine buffer (section 2). More than 95% of the radioactivity applied was eluted together with cytochrome bc_1 and well separated from any free cytochrome *c*. A 1:1 complex was also formed under equilibrium conditions [10] when 6 nmol cytochrome bc_1 was chromatographed on a column of Sephadex G-75 equilibrated with buffer containing 10 μM [^{3}H]-methylated cytochrome *c*.

Differential acetylation and methylation were used to detect altered chemical reactivity of ε-amino groups in cytochrome *c* bound to cytochrome bc_1. The reactivity differences were quantitated by a shielding factor as described in section 2 and detailed [3]. Results from two experiments where equimolar amounts of cytochromes *c* and bc_1 were acetylated in the presence and absence of 0.25 M NaCl are shown in fig.1. Lysine residue 13 was 4.75-times less reactive in the bc_1-bound cytochrome *c*. Similarly, the groups of residues in positions 5, 7, 8, 72–73 and 86–88 were 2.6-, 2.9- and 3.5-times less reactive, respectively, in the bound cytochrome *c*. But the remaining 8 lysine residues were equally reactive or only marginally less reactive in the complex. No shielding factors were determined for lysine residues 60 and 79.

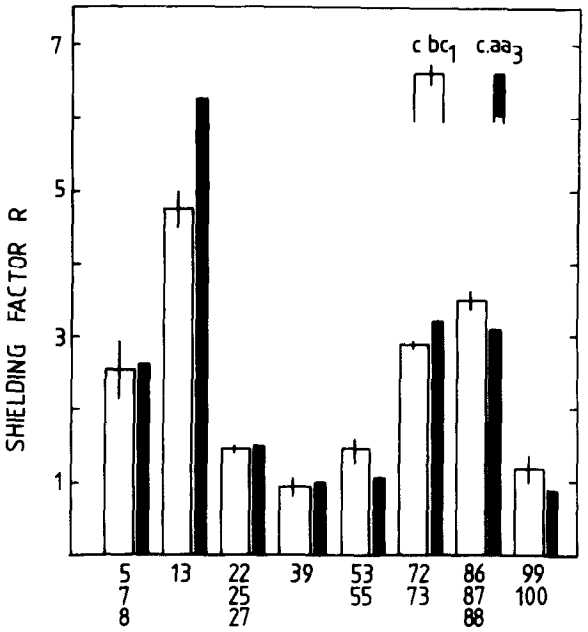

Fig.1. Effect of cytochrome bc_1 and of cytochrome oxidase (aa_3) on the rate of acetylation of lysine residues in cytochrome *c*. The rate of acetylation of one or a group of 2–3 residues is reduced by *R* (shielding factor) in the complexes. Each bar corresponds to one or two of the following peptides which corresponds to one or two of the following peptides which contain lysine residues in the sequence positions indicated below the bars: 1–8, 11–13, 21–32, 22–34, 36–45, 45–56, 49–56, 68–73, 85–88, 98–100 and 98–104 (sequence position of N- and C-terminal amino acid is given for each peptide). Isolation, purification and amino acid composition of the peptides have been described [3]. The height of each open bar corresponds to the average *R* from 2 experiments with the cytochrome *c*: cytochrome bc_1 complex (inactive bc_1), the vertical line indicating the range of *R*-values. Closed bars show the corresponding *R*-values from 4 experiments with the cytochrome *c*: cytochrome oxidase complex (1:1 stoichiometry) as in [3], normalized to $R = 1.00$ for residue 39 (see text for explanation).

Also included in fig.1 are the shielding factors from experiments with the 1:1 complex of cytochrome *c* with cytochrome oxidase. Except for residue 13 all *R*-values were very similar for the two different complexes. *R*-values from the complex with the oxidase were normalized to $R = 1.00$ for residue 39. Normalization was necessary because in the former experiments cytochrome *c* had been labeled in the presence and absence of oxidase.

Therefore each lysine residue had an R-value > 1 since the oxidase was competing for the small amount of ^{3}H-labeled reagent in only one of the paired experiments. Uniformly lower ^{3}H-labeling was observed in the presence of oxidase concurrent with the specific shielding of particular residues [3]. The present experimental procedure including cytochrome bc_1 in the paired experiments but changing the ionic strength instead is superior since under these conditions the R-values signal directly the shielding effect of cytochrome bc_1. In order to prove that the change in the ionic strength by itself does not influence the reactivity of the lysine residues cytochrome c alone was acetylated with or without 0.25 M NaCl. R-values around 1 were obtained for all of the peptides shown in fig.1, the average value being 1.08 ± 0.17.

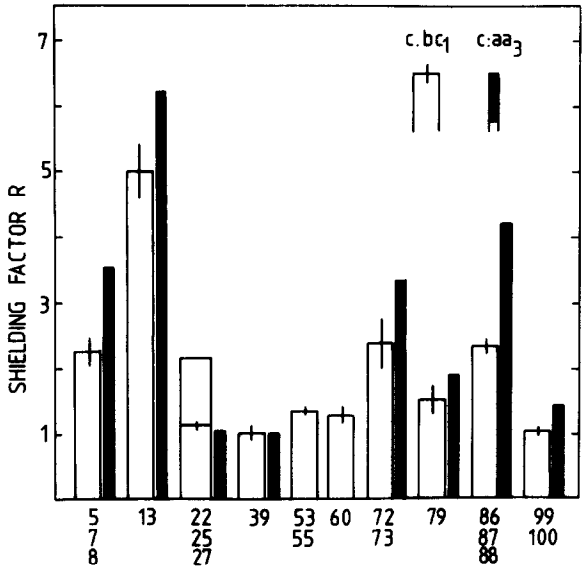

Fig.2. Effect of cytochrome bc_1 and of cytochrome oxidase (aa_3) on the rate of reductive methylation of lysine residues in cytochrome c. Open bars show the average R from 3 experiments with cytochrome bc_1. Two experiments were performed with inactive and one with active cytochrome bc_1. The higher R-value for residues 22, 25, 27 is from the experiment with active cytochrome bc_1. Closed bars are from a single experiment with cytochrome oxidase (1:1 complex) reported in [3]. R-values are normalized to R = 1.00 for residue 39. The following peptides were analyzed: 1–8, 11–19, 20–27, 20–31, 35–40, 36–40, 49–56, 57–63, 68–73, 74–80, 85–88 and 98–100. See fig.1 and the text for further explanations.

Differential methylation of the c:bc_1 complex gave results very similar to the acetylation (fig.2). Cytochrome c was [^{3}H]methylated with or without addition of cytochrome bc_1 and therefore the R-values were normalized to 1.00 for residue 39. The actual shielding factor was 1.73 for this residue. Lysine residue 13 was again the most shielded followed by residues 5, 7, 8, 72–73, and 86–88. The remaining lysine residues remained about equally reactive in free and bc_1-bound cytochrome c. However when the experiment was performed with an active preparation of cytochrome bc_1 [7] the group of residues 22, 25, 27 was distinctly less reactive in the complex. The shielding factors from the complex with the oxidase follow a similar overall pattern but R-values were higher for the less reactive residues in the cytochrome c: cytochrome oxidase complex.

4. Discussion

The differential labeling technique as applied here, and in [11,12], revealed that particular groups of lysine residues of cytochrome c are less reactive in the complex with cytochrome oxidase [3]. We now find that the very same groups of residues are less reactive in a cytochrome c: cytochrome bc_1 complex. The lysine residues of quenched reactivity demarcate a common binding domain which stretches over a contiguous area from the top right of the molecule (residues 5, 7, 8) over the entrance to the heme crevice (residue 13) to the top left (residues 86–88) and down to the middle of the left-hand side (residues 72, 73; see [13] for top and front view of cytochrome c).

The question remains if the two in vitro complexes represent the functional complexes in the inner mitochondrial membrane. There is little doubt for the complex with the oxidase because of the very similar binding site deduced from kinetic experiments with singly substituted cytochrome c derivatives [4,5]. The significance of the c:bc_1 complex is less clear. Different shielding of residues 22, 25, 27 by active and inactive bc_1 points to somewhat different binding modes (fig.2). Experimental indications for a site of reaction for bc_1 which differs from that for the oxidase have been reported and interpreted as suggestive for a 'static' mechanism [4,14–16,19].

Electron transfer from and to heme *c* can be discriminated by site-specific anti-cytochrome *c* antibodies [14]. Selective chemical modification of amino acid side chains in cytochrome *c* affects oxidation and reduction of heme *c* to different degrees, but some results are equivocal [15–17].

Further, cytochrome *c* seems to accept electrons from c_1 while still bound to the oxidase since the turnover of electrons to oxygen is faster than the rate of dissociation of cytochrome *c* from the high affinity binding site on the oxidase [4,18,19]. There are several explanations for these discrepancies. For example, the binding site and the site of reaction need not be the same [14], or the two binding sites might differ at domains which cannot be discerned by our approach for lack of suitably positioned lysine residues. Nevertheless we must conclude that the mitochondrial proteins donating and accepting electrons to and from cytochrome *c* can bind to an extended common surface domain of the cytochrome *c* molecule. This observation is compatible with a mobile mode of action of cytochrome *c* during electron transport but is not easily rationalized by one and the same molecule binding simultaneously to cytochrome bc_1 and to the oxidase.

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