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BINDING OF CYTOCHROME c TO THE CYTOCHROME bc_1 COMPLEX (COMPLEX III) AND ITS SUBUNITS CYTOCHROME c_1 AND b[1]

H.R. Bosshard*[2], M. Zürrer*, H. Schägger** and G. von Jagow**

* Biochemisches Institut der Universität, CH-8028 Zürich, Switzerland

** Institut für Physiologische Chemie und Physikalische Biochemie der Universität, Goethestrasse 33, D-8000 München 2 (G.F.R.)

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SUMMARY. Cytochrome c_1, the electron donor for cytochrome c, is a subunit of the mitochondrial cytochrome bc_1 complex (complex III, cytochrome c reductase). To test if cytochrome c_1 is the cytochrome c-binding subunit of the bc_1 complex, binding of cytochrome c to the complex and to isolated cytochrome c_1 was compared by a gel-filtration method under non-equilibrium conditions (a bc_1 complex lacking the Rieske iron-sulfur protein was used; von Jagow et al. (1977) Biochim. Biophys. Acta 462, 549-558). The approximate stoichiometries and binding affinities were found to be very similar. Binding of cytochrome c to isolated cytochrome b which is another subunit of the reductase was not detectable by the gel-filtration method. Further, the same lysine residues of cytochrome c were shielded towards chemical acetylation in the complexes $c:c_1$ and $c:bc_1$. From this we conclude that the same surface area of cytochrome c is in direct contact with cytochrome bc_1 and with cytochrome c_1 in the respective complexes and that therefore cytochrome c is most probably the structural ligand for cytochrome c in mitochondrial cytochrome c reductase.

Cytochrome c mediates the electron transfer between mitochondrial cytochrome c reductase (complex III, cytochrome bc_1 complex) and cytochrome c oxidase (complex IV) in the final segment of the mitochondrial electron transport chain. To understand the molecular mechanism of this process we have to

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[2] To whom to address correspondence.

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know the structural components of the multi-subunit complexes III and IV and of cytochrome c which interact with each other during the electron transfer process. The binding site on cytochrome c for the complexes III and IV is known in detail (1-6) but there are equivocal reports about the cytochrome c-binding subunits of the oxidase (7-9). As for the reductase, the redox potential of the subunit cytochrome c_1 is nearest to that of cytochrome c (10) and a specific high affinity $c:c_1$ complex has been isolated (11). Cytochrome c_1 might therefore be the functional as well as the immediate structural reaction partner of cytochrome c. Our experiments presented here strengthen this proposition.

MATERIALS AND METHODS. Cytochrome bc_1[3] and cytochrome b were prepared as described (12,13). Cytochrome c_1 was recovered from the same hydroxyapatite column used to purify cytochrome b (13). The tightly bound cytochrome c_1 could be released from this column with 350 mM sodium phosphate, 0.5% Triton X 100, pH 7.2, following the elution of cytochrome b (see ref. 13 for details). The material was 65% cytochrome c_1 by weight as judged from protein content and from spectroscopic analysis assuming a molecular weight of 30'000 and using ε(552.5-540, red-ox) = 17.5 mM^{-1} cm^{-1}. Polyacrylamide gel electrophoresis showed contamination with at least two low molecular weight subunits of cytochrome bc_1 and some inhomogeneity in the main peak (Fig. 1). - The source of all other materials was as before (3,4).

Binding studies were done at 4° C on a column (0.9 x 19 cm) of Sephadex G-75 superfine (Pharmacia) in 10 mM triethanolamine·HCl, 0.2% Tween-80, pH 8.0, and with 0, 20 or 40 mM NaCl. The sample (0.1 ml) contained 5 to 25 nmol of cytochrome c (horse heart) or [^{3}H]methylated cytochrome c (ref. 3, approx. 40'000 cpm/nmol, average degree of methylation below one methyl group per cytochrome c molecule) together with 5 nmol of cytochrome c_1 or cytochrome bc_1. Ascorbate if present was 1 mM. Given the amount of cytochrome c_1 in the sample, the stoichiometry of the eluted complexes was calculated from (a) the difference between cytochrome c added to the sample and recovered as free cytochrome c (concentration based on ε(550, red) = 29 mM^{-1} cm^{-1}) and from (b) the distribution of the radio-

[3] Cytochrome bc_1 is defined here as complex III lacking the Rieske iron-sulfur protein as described in ref. 12. The concentration of cytochrome bc_1 is expressed as its content of cytochrome c_1 calculated from the ascorbate-reduced minus ferricyanide-oxidized difference spectrum.

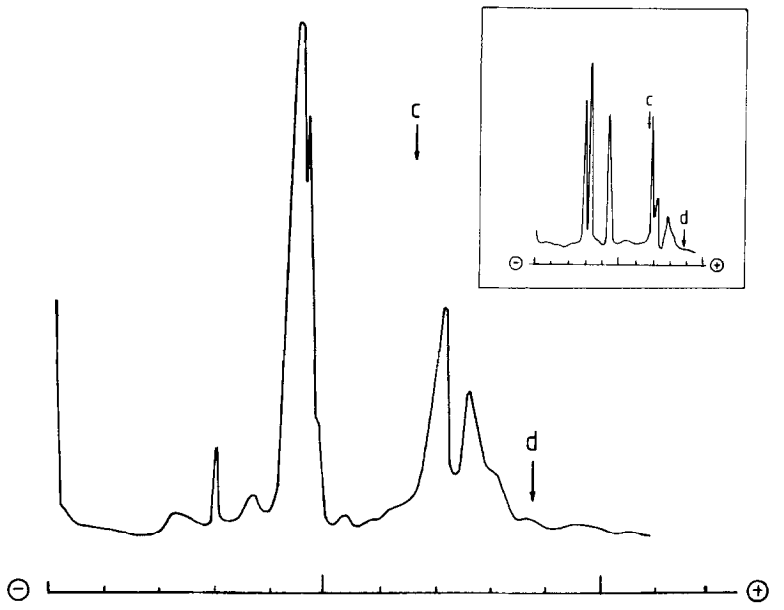

Figure 1. Densitometric tracings of dodecyl sulfate polyacrylamide gels of cytochrome $\underline{c}_1$ (20 µg total protein) and, in the inset, of cytochrome $\underline{bc}_1$ (5 µg total protein). Gels consisted of a 10% to 24% linear gradient of polyacrylamide topped by a 3% stacking gel and were run in the buffer system of Laemmli (19). The migration distances of cytochrome $\underline{c}$ and bromophenol-blue dye are indicated by arrows c and d, respectively.

activity in the complex and in free cytochrome $\underline{c}$ (Fig. 2). The two methods gave similar results (Table 1).

Differential acetylation of the lysine residues of cytochrome $\underline{c}$ was performed as in ref. 3. Briefly, cytochrome $\underline{c}$ and $\underline{c}_1$ (both 50 µM, ferri-form) were trace-labeled (3,14) with [^{3}H]acetic anhydride at 0° C in parallel experiments in (a) 20 mM triethanolamine·HCl, 0.2% Tween-80, pH 8.0 (a stable $\underline{c}$:$\underline{c}_1$ complex is formed under these conditions), (b) the same buffer plus 0.25 m NaCl (cytochromes dissociated). The ^{3}H-labeled derivatives were mixed with equimolar amounts of [^{14}C]acetylated cytochrome $\underline{c}$ (3) and fully acetylated with cold acetic anhydride in excess. The chemically homogeneous but isotopically heterogeneous derivatives from (a) and (b) above were proteolytically cleaved and the labeled peptides purified by chromatography/electrophoresis. The ^{3}H/^{14}C ratio of a lysine residue (or residues) labeled in free cytochrome $\underline{c}$ divided by the ^{3}H/^{14}C ratio of the same residue(s) labeled in the $\underline{c}$:$\underline{c}_1$ complex is called 'shielding factor' R. R > 1 indicates reduced reactivity of lysine residues in the complex.

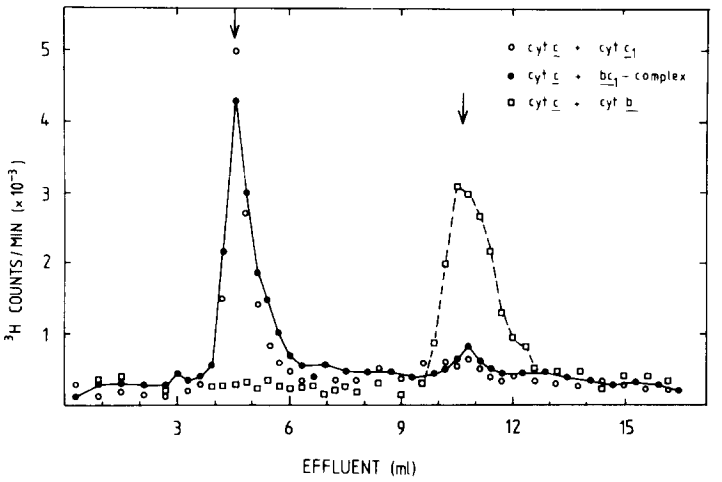

Figure 2. Detection by gel-filtration of binding of [^{3}H]methylated cytochrome c to cytochrome c_1 and to cytochrome bc_1. 1 mol cytochrome c/mol cytochrome c_1 was applied to the column. [^{3}H]methylated cytochrome c (15 nmol) and cytochrome b (5 nmol) in a sample volume of 0.15 ml was chromatographed on the same column. Elution was with buffer containing 20 mM NaCl (see Materials and Methods). The arrows indicate the void volume and the elution volume for free cytochrome c.

RESULTS AND DISCUSSION. The in vitro complexes between cytochrome c and cytochrome c_1 or cytochrome bc_1[3] can be isolated on a column of Sephadex G-75 where they move with the void volume whereas free cytochrome c is retarded (Fig. 2 and ref. 11). More than 0.9 mol of cytochrome c appears in the complex if the sample contains 1 mol cytochrome c/mol cytochrome c_1. From this a dissociation constant $K_d \leq 10^{-7}$ M is estimated according to Dixon (15). In contrast, no complex between cytochromes c and b can be detected on the same column hence K_d must be $\geq 10^{-5}$ M (Fig. 2). Increasing the ratio of cytochrome c to c_1 in the sample increases the stoichiometry of the eluted complex. Table I indicates that at low ionic strength at least 2 mol of cytochrome c firmly bind to cytochrome c_1 or to cytochrome bc_1. Cytochromes c and c_1, but not b, are reduced by ascorbate. Association between the reduced cytochromes seems to be somewhat weaker.

The gel-filtration method applied here provides a very approximate measure only of the stoichiometry and strength of binding.

TABLE I: Binding of cytochrome c to cytochrome c_1 and to cytochrome bc_1[3] as detected by gel-filtration under non-equilibrium conditions.

$\frac{\text{cytochrome c}}{\text{cytochrome } bc_1}$ in applied sample	in eluted complex (1)	(2)	$\frac{\text{cytochrome c}}{\text{cytochrome } c_1}$ in applied sample	in eluted complex (1)	(2)
1.0	0.92	not.det.	1.0	< 0.95	< 0.95
1.0 (ascorbate)	0.95	0.82	1.0 (ascorbate)	0.95	0.83
2.0	1.38	1.02	2.0	1.64	1.42
2.0 (ascorbate)	1.05	0.91	2.0 (ascorbate)	0.92	0.95
3.6	1.63	1.75	5.0	1.47	1.62
3.6 (ascorbate)	1.38	1.40	5.0 (ascorbate)	1.12	1.08
3.6 (no NaCl)	2.24	2.46	5.0 (no NaCl)	2.25	not.det.
3.6 (40 mM NaCl)	0.16	< 0.1	5.0 (40 mM NaCl)	< 0.1	< 0.1

Values in column (1) are from the distribution of ^{3}H-radioactivity and in column (2) from absorbance at 550 nm. The buffer contained 20 mM NaCl unless indicated. See Materials and Methods for details.

Weiss and Juchs (16) have studied in detail the association between cytochrome c and cytochrome bc_1 from Neurospora crassa. At pH 8.0 they found the affinity and stoichiometry to depend on ionic strength (K_d's between > 5 x 10^{-8} M and 3.7 x 10^{-6} M, 1 to 3 binding sites) and significantly weaker binding in the presence of ascorbate.

Table 1 reveals no obvious difference in the binding of cytochrome c to cytochrome bc_1 and to isolated cytochrome c_1, in agreement with the suggested role of cytochrome c_1 as the ligand for cytochrome c. Further evidence is the close similarity of the binding areas for cytochrome bc_1 and for cytochrome c_1 on the surface of the cytochrome c molecule. The protein-protein contact areas of the two complexes were studied by differential chemical modification, a procedure measuring altered chemical reactivity of particular amino acid side chains following complex formation (14). The results are shown in Fig. 3. Lysine residue 13 and the groups of residues in sequence positions 72-73 and 86-88 are from 2 to 4 times less reactive in the cytochrome c_1-bound molecule whereas the remaining lysine residues are of the same ($R = 1 \pm 0.3$) or only marginally reduced reactivity (residues 5,7,8) in bound cytochrome c. (No shielding factor was obtained for lysine 79). Also included in Fig. 3 are the shielding factors from experiments with the 1:1 complex of cytochrome c with cytochrome bc_1 (4). Obviously, the same lysine residues are shielded whether cytochrome c binds to isolated cytochrome c_1 or to the whole of complex III (the pattern of shielding factors obtained with cytochrome bc_1 or with complex III is the same, ref. 4). Comparison of individual R-values shows, however, that the chemical reactivity of residue 13 is less and that of the group of residues 86-88 is more affected in the $c:c_1$ complex than in the $c:bc_1$ complex. Residues 5,7-8 and 72-73 are significantly less shielded in the $c:c_1$ complex.

The lysine residues shielded by complexes III and IV are identical (3,4) and are spread over a contiguous surface area at the upper front and around the top entrance to the heme cleft of the molecule (conventional top and front view

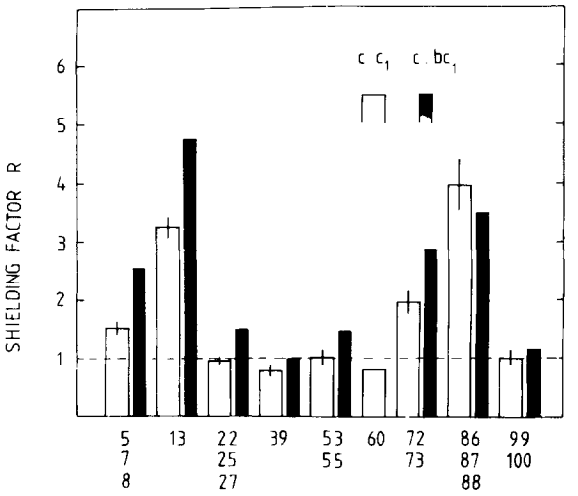

Figure 3. Effect of cytochrome c_1 and of cytochrome bc_1 on the rate of acetylation of lysine residues in cytochrome c. The rate of acetylation of one or of a group of 2-3 residues is reduced by R in the complexes $c:c_1$ (1:1, open bars) and $c:bc_1$ (1:1, see footnote[3], closed bars). Each bar corresponds to one or two of the following peptides which contain lysine residues in the sequence positions indicated below the bars: 1-8, 11-13, 22-34, 36-45, 49-56, 68-73, 85-88,85-94, 98-100, and 98-104 (sequence position of N- and C-terminal residue of peptides given). Isolation, purification and amino acid composition of these peptides were as described (3). The height of the open bars corresponds to the average R from 2 experiments with the $c:c_1$ complex. R-values represented by the closed bars are the average from 4 experiments performed with the $c:bc_1$ complex and are taken from ref. 4. Vertical lines indicate the range of single R-values.

according to ref. 17). Since residues 5,7-8 and 72-73 are at the periphery of the binding site defined for complex III and IV and since they show significantly lower R-values in the $c:c_1$ complex, we assume that binding of cytochrome c_1 is centered to a small area just to the left of the top entrance to the heme cleft and near the positive end of the molecular dipole axis of cytochrome c (18,6).

Cytochrome c_1 used in the differential acetylation experiment was contaminated with small molecular weight subunits of cytochrome bc_1 (Fig. 1). We cannot exclude so far that these proteins are not also part of the complex. Since cytochrome c_1 has at least two binding sites, binding might take place at

both sites even in the formal 1:1 complex unless the K_d's for the two sites differ widely. Moreover, we do not know with certainty whether we are dealing with the functionally active complexes. However, kinetic analysis of monosubstituted cytochrome c derivatives shows that chemical modification of the very same lysine residues found to be shielded by cytochrome c_1 and complex III increases the K_m-values for the reduction by isolated complex III (6). The order of increasing K_m-values of monosubstituted derivatives (Lys 72 < Lys 13 < Lys 86,87, ref. 6) even follows the order of increasing shielding factors for the $c:c_1$ complex (Fig. 3). We therefore feel justified to regard cytochrome c_1 as the binding component for cytochrome c in isolated complex III and possibly also in the intact mitochondrial electron transfer chain.

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