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BINDING OF CYTOCHROME c_2 TO THE ISOLATED REACTION CENTER OF *RHODOSPIRILLUM RUBRUM* INVOLVES THE "BACKSIDE" OF CYTOCHROME c_2

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SUMMARY. Lys 109, Lys 112 and Glu 1 of cytochrome c_2 from *Rhodospirillum rubrum* G-9 are about 4-fold less reactive towards acetic anhydride when cytochrome c_2 is bound to the isolated photosynthetic reaction center from the same organism. The three shielded residues are clustered together on the "backside" of cytochrome c_2. This contrasts with mitochondrial cytochrome c where "frontside" lysines are protected by different physiological electron transfer partners. © 1985 Academic Press, Inc.

INTRODUCTION - Cytochrome c_2 is an electron carrier involved in cyclic electron transport in purple bacteria. In many species it provides an electron to the photosynthetic reaction center, subsequent to the primary photochemical process (1). This function corresponds to the electron transfer from mitochondrial cytochrome c to cytochrome c oxidase. Indeed, mitochondrial cytochrome c can substitute for cytochrome c_2 at the reaction center (2).

The electron transfer interaction domain of mitochondrial cytochrome c is known in some detail (3-6 and references therein, review in ref. 7). In view of the remarkable similarity of cytochrome c_2 of bacterial origin to mitochondrial cytochrome c (8, 9) we began to characterize the surface domain of cytochrome c_2 that is recognized by the reaction center in the isolated

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cytochrome c_2 - reaction center complex. We used differential chemical modification of lysine residues, by which method the electron transfer domain of mitochondrial cytochrome c had been mapped previously (6, 7). With this method the surface area involved in interaction is deduced from the decrease in reactivity of those lysines that are partially shielded in the electron transfer complex.

MATERIALS AND METHODS - Rhodospirillum rubrum (carotenoidless mutant G-9) was grown and reaction centers were isolated essentially as before (10) except that Tris buffer was replaced by 10 mM triethanolamine-HCl (pH 8.2). The reaction center preparation had A_{280}/A_{802} = 1.43 after chromatography on Sepharose 6B and concentration by ultrafiltration. The concentration was calculated using ε_{802} = 288 mM^{-1} cm^{-1} (value for reaction centers from Rps. sphaeroides, ref. 11). Cytochrome c_2 was purified from R. rubrum (12). Purity was checked by amino acid analysis and SDS polyacrylamide gel electrophoresis. All other materials were as before (6).

Binding of cytochrome c_2 to the reaction center was tested by chromatography of mixtures of the two proteins on a column (0.7 x 23 cm) of Biogel P-100 (BioRad), equilibrated and eluted (1.7 ml/hr) with the buffer used in differential acetylation (see below). K_d was calculated from the ratio of cytochrome c_2 to reaction center in the front peak (13). The ratio was determined by multicomponent analysis on a Hewlett-Packard 8450A spectrophotometer (14).

Differential acetylation was performed as before (6). Briefly, ferricytochrome c_2 (88 μM) and reaction centers (120 μM) in 10 mM triethanolamine-HCl, 0.025 % lauryldimethylamineoxide, pH 8.2, were reacted at 0° C with [^{3}H]acetic anhydride (0.6 mM, 7.5 Ci/mmol). In a parallel experiment, 0.25 M NaCl was added to dissociate the complex, all other conditions being the same. [^{3}H]Acetylated cytochrome c_2 was separated from the reaction center on a column (0.9 x 30 cm) of Ultrogel AcA54, equilibrated and eluted with the same buffer used in acetylation of free cytochrome c_2. ^{3}H-labeled cytochrome c_2 from the two parallel experiments had less than 0.5 mol [^{3}H]acetyl group per mol of protein, on average. A low degree of labeling guarantees that R-values (see below) monitor the influence of complex formation on chemical reactivity (6). Equimolar amounts of the two derivatives (200 nmol) were each mixed with equimolar amounts of uniformly [^{14}C]acetylated cytochrome c_2 derivatives (500 nmol, 8 Ci/mol). The mixtures were treated with non-radioactive acetic anhydride in excess. The derivatives were digested by thermolysin, chymotrypsin and protease from Staph. aureus V8, respectively. Labeled peptides were separated by high-performance liquid chromatography on an Aquapore RP-300 reversed phase column using acetonitrile gradients in 0.1 % trifluoroacetic acid (equipment as in ref. 15). Purified peptides were analyzed for amino acid composition, N-terminus and ^{3}H/^{14}C radioactivity ratios. Peptides with several lysines were degraded by a modified Edman method (16) to get ^{3}H/^{14}C ratios of single N^{ε}-acetyllysines.

The ^{3}H/^{14}C ratio of a N^{ε}-acetyllysine residue labeled in free

cytochrome $\underline{c}_2$ was divided by the $^3H/^{14}C$ ratio of the same residue labeled in complexed cytochrome $\underline{c}_2$. The resulting number is called reactivity ratio R (6).

The following peptides were analyzed (sequence from ref. 9, position of acetylated residue): 1-5, Glu 1; 1-8, Glu 1; 9-37, Lys 9, 12, 13; 21-33, Lys 27; 38-50, Lys 43; 40-48, Lys 43; 55-56, Lys 56; 56-60, Lys 56, 58; 57-59, Lys 58; 71-76, Lys 72, 75; 81-100, Lys 81, 86, 88, 90; 93-94, Lys 94; 94-103, Lys 94, 97; 95-103, Lys 97; 109-112, Lys 109, 112; 108-110, Lys 109; 111-112, Lys 112. When the same N^{ε}-acetyllysine was obtained from different peptides the $^3H/^{14}C$ ratios agreed within ± 15 % or better. Average R-values are presented for these residues (Table I).

RESULTS - Cytochrome $\underline{c}_2$ and purified reaction center form a complex at low ionic strength (17, and references therein). In Rps. sphaeroides the complexes with both cytochrome $\underline{c}_2$ and mitochondrial cytochrome c show 1:1 stoichiometry and a dissociation constant of about 1 μM (17). Binding of cytochrome $\underline{c}_2$ to the reaction center from R. rubrum under the conditions of the acetylation experiment was confirmed by chromatography of mixtures of the two proteins on Biogel P-100 under non-equilibrium conditions. Assuming a 1:1 complex, the dissociation constant was 1 - 2 μM.

Acetylation of the complex with [^{3}H]acetic anhydride was performed at 6 mM ionic strength with the reaction center in excess over cytochrome $\underline{c}_2$. Assuming $K_d < 2$ μM, > 95 % of cytochrome $\underline{c}_2$ was bound to the reaction center, on average. Acetylation of free cytochrome $\underline{c}_2$ was achieved at 0.25 M ionic strength, with the reaction center present in order to keep constant the total amount of reactive amino groups (complex dissociated). The degree of ^{3}H-acetylation of each amino group of free and complexed cytochrome $\underline{c}_2$ was calculated from $^3H/^{14}C$ ratios. A reactivity ratio R was defined so that R = 1 indicates equal, R > 1 reduced and R < 1 increased reactivity in complexed cytochrome $\underline{c}_2$.

Control experiments with and without 0.25 M NaCl were performed in the absence of the reaction center to see whether NaCl

TABLE I: R-VALUES AND R_{NaCl}-VALUES OF AMINO GROUPS

Residue	R-value[1]	R_{NaCl}-value[1]	R-value (corrected)[2]
Glu 1	4.26	1.35	3.91
Lys 9	1.37	1.11	1.26
Lys 12	1.27	1.16	1.11
Lys 13	0.88	0.80	1.08
Lys 27	1.37	1.12	1.25
Lys 43	0.46	1.11	0.35
Lys 56	1.72	2.04	0.68
Lys 58	2.28	2.33	0.95
Lys 72	0.83	1.04	0.79
Lys 75	0.99	0.79	1.20
Lys 81	2.31	2.58	0.73
Lys 86	0.80	1.09	0.71
Lys 88	0.70	1.04	0.66
Lys 90	0.74	1.04	0.70
Lys 94	1.15	0.82	1.33
Lys 97	1.62	1.04	1.58
Lys 109	4.41	1.16	4.25
Lys 112	4.15	1.25	3.90

[1] See text for explanation

[2] Correction for effect of 0.25 M NaCl is R-value (corrected) = R-value - R_{NaCl}-value + 1.

changes the reactivity of amino groups. The reactivity ratio R_{NaCl} refers to the control experiments.

Table I summarizes the results. In the last column, the R-values corrected for the effect of NaCl are shown. Correction is small except for residues 56, 58 and 81. Most residues are about equally reactive in free and complexed cytochrome $\underline{c}_2$; residue 43 is more reactive in the complex. The amino groups of Glu 1, Lys 109 and Lys 112 are 4-times less reactive in complexed cytochrome $\underline{c}_2$. If the molecule is oriented with the exposed heme edge facing the viewer these three residues are grouped together on the backside, opposite to the ring of positively charged lysines surrounding the heme entrance (Fig. 1). The more reactive Lys 43 is also found on the backside.

DISCUSSION - The domain of electron transfer interaction of mitochondrial cytochrome $\underline{c}$ is on the front surface of the

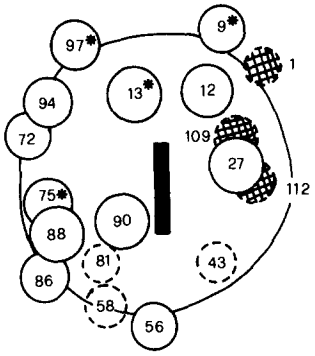

Fig. 1: Schematic front view of cytochrome c_2. The heme is viewed edge on (solid bar). Circles give the position of free amino groups of the protein. Cross-hatched circles indicate amino groups with lower reactivity in the cytochrome c_2 - reaction center complex. Amino groups corresponding to those at the electron transfer interaction domain of mitochondrial cytochrome c are marked by a star.

molecule. Lysines 8, 13, 72, 86 and 87 (horse cytochrome c) are part of the interaction domain which includes the solvent accessible edge of the heme group, indicating that electron exchange probably takes place at the heme edge or via a structure close to it (7). The 3D structure of cytochrome c_2 is very similar to that of mitochondrial cytochrome c (8, 9). Again, the heme edge is accessible at the molecular surface and is surrounded by several lysines. Residues of cytochrome c_2 corresponding to the "active site" of mitochondrial cytochrome c are Lys 9, 13, 75 and 97 (Fig. 1). They should be protected by the reaction center if binding follows the mitochondrial pattern. This is not the case. Instead, three less reactive residues are found on the backside of the molecule, far removed from the heme center. This result can be interpreted in different ways.

First, the isolated complex might differ from that in the intact photosynthetic membrane. However, the isolated complex was competent in electron transfer under steady state conditions with either cytochrome c_2 or mitochondrial cytochrome c (2, 18; V. Wiemken, unpublished experiments).

Alternatively, binding to the backside is not effective but the complex is in rapid equilibrium with a minor form competent for electron transfer. Or else, no lysines are protected by the reaction center, yet three residues outside of the interaction domain become less reactive by a conformational change.

Finally, the domain at the backside could indeed constitute the electron transfer domain of cytochrome $\underline{c}_2$. This possibility has some speculative support. For example, cytochrome $\underline{c}_2$ is a poor electron donor for mitochondrial cytochrome $\underline{c}$ oxidase but a good one for mitochondrial reductase, indicating different modes of interaction of cytochrome $\underline{c}_2$ and mitochondrial cytochrome $\underline{c}$ (3). Further, the negative end of the molecular dipole of cytochrome $\underline{c}_2$ emerges about 5 Å from Lys 112 at the back of the molecule, the overall direction and magnitude of the dipole being not much different from that of mitochondrial cytochrome $\underline{c}$ (W.H. Koppenol, personal communication). Therefore, a mechanism of electron transfer similar to that proposed for mitochondrial cytochrome $\underline{c}$ seems possible (19). The dipole moment causes cytochrome $\underline{c}_2$ to orient itself in the electric field of the reaction center prior to electron transfer. Cytochrome $\underline{c}_2$ would have to interact with a positively charged domain on the reaction center, as opposed to negatively charged domains recognized by mitochondrial cytochrome $\underline{c}$ (6, 20, 21; but see 2).

It will be most important to define the domain of mitochondrial cytochrome $\underline{c}$ that is recognized by the reaction center and to map the binding site of cytochrome $\underline{c}_2$ for mitochondrial reductase, a competent electron donor of cytochrome $\underline{c}_2$ (3). Such studies are under way in our laboratory. In addition, more elaborate cross-linking studies (22) and kinetic experiments with derivatives of cytochrome $\underline{c}_2$ should help to corroborate or dis-

prove the proposed electron transfer domain on the backside of cytochrome c_2.

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