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Interaction of Horse Cytochrome *c* with the Photosynthetic Reaction Center of *Rhodospirillum rubrum*

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Abstract

Mitochondrial cytochrome *c* (horse), which is a very efficient electron donor to bacterial photosynthetic reaction centers *in vitro*, binds to the reaction center of *Rhodospirillum rubrum* with an approximate dissociation constant of 0.3–0.5 μM at pH 8.2 and low ionic strength. The binding site for the reaction center is on the frontside of cytochrome *c* which is the side with the exposed heme edge, as revealed by differential chemical acetylation of lysines of free and reaction-center-bound cytochrome *c*. In contrast, bacterial cytochrome c_2 was found previously to bind to the detergent-solubilized reaction center through its backside, i.e., the side opposite to the heme cleft [Rieder, R., Wiemken, V., Bachofen, R., and Bosshard, H. R. (1985). *Biochem. Biophys. Res. Commun.* **128**, 120–126]. Binding of mitochondrial cytochrome *c* but not of mitochondrial cytochrome c_2 is strongly inhibited by low concentrations of poly-L-lysine. The results are difficult to reconcile with the existence of an electron transfer site on the backside of cytochrome c_2.

Key Words: Cytochrome *c*; cytochrome c_2; reaction center; acetylation; lysine; binding site; *Rhodospirillum rubrum*.

Introduction

The 3D structures of mitochondrial cytochrome *c* and bacterial cytochrome c_2 are very similar; they both feature the "cytochrome *c*-fold" and a very asymmetrical distribution of surface charges (Salemme, 1977). The electron-transfer interaction domain of mitochondrial cytochrome *c* is located at an area which includes the solvent-accessible heme edge at the molecule's

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frontside and part of the surface on top of, and slightly to the left of, the frontside (Margoliash and Bosshard, 1983; see Salemme, 1977, for standard view of cytochrome *c*). The interaction domain is virtually the same for all the redox partners tested. In view of the remarkable similarity between cytochrome *c* and cytochrome c_2, it was expected that cytochrome c_2 contains a similar electron transfer interaction domain on its frontside. Instead, we recently found that in the cytochrome c_2–reaction center complex three primary amino groups on the backside of cytochrome c_2 are partially shielded from reaction with acetic anhydride, indicating a binding site for the reaction center on the backside, opposite to the exposed heme edge (Rieder *et al.*, 1985).

It is a matter of debate whether the area at the backside constitutes a genuine electron transfer domain of cytochrome c_2. Since mitochondrial cytochrome *c* is a very efficient *in vitro* electron donor to the bacterial reaction center (Rickle and Cusanovich, 1979; Nicholls, 1974), the analysis of the mode of binding of mitochondrial cytochrome *c* to the reaction center would constitute an indirect test of the feasibility of an electron-transfer domain on the backside of structurally related cytochrome c_2. Here we show that such backside interaction cannot be observed with mitochondrial cytochrome *c*.

Experimental

R. rubrum (carotenoid-less mutant G-9) was grown and reaction centers isolated and assayed as before (Rieder *et al.*, 1985; Snozzi and Bachofen, 1979). Cytochrome c_2 was purified from *R. rubrum* (Sponholtz *et al.*, 1976). Horse cytochrome *c* type III, rechromatographed on CM-Sephadex (Brautigan *et al.*, 1978), and poly-L-lysine (M_r 15,000–30,000) were from Sigma.

Binding of cytochromes to the reaction center was determined by chromatography of a mixture of the two proteins (0.2 ml) on a column of Biogel P100 (0.7 × 23 cm, BioRad), equilibrated and eluted (1.7 ml/h) at 4°C in the dark with 10 mM triethanolamine-HCl and 0.025% lauryldimethylamineoxide, pH 8.2. Fractions of 0.36 ml were analyzed for cytochrome and reaction center by multicomponent analysis on a Hewlett-Packard 8450A diode-array spectrophotometer (Schleifer and Willis, 1980). The dissociation constant was estimated from $K_d = (Y - \ln Y - 1)CA/V$, where Y = moles cytochrome per mole reaction center in the front peak, C = initial concentration of reaction center in the sample, A = sample volume, and V = elution volume between front peak and peak containing excess unbound cytochrome (Dixon, 1976).

Differential chemical acetylation was conducted as before (Bosshard, 1979; Rieder and Bosshard, 1980; Bosshard *et al.*, 1986). For trace-labeling

with [^{3}H]acetic anhydride, cytochrome *c* and reaction center were dissolved in the same buffer used in binding experiments. Initial concentrations were 45.3 μM reaction center, 32.6 μM cytochrome *c*, and 0.25 mM [^{3}H]acetic anhydride. Labeling of free cytochrome *c* was performed in the presence of 250 mM NaCl, all other conditions being equal. ^{3}H-labeled cytochrome *c* was separated from reaction center on CM-cellulose (Bosshard *et al.*, 1986). The derivatives obtained from labeling free and reaction-center-bound cytochrome *c* were fully acetylated with excess nonradioactive anhydride under denaturing conditions and mixed with equimolar amounts of fully ^{14}C-acetylated cytochrome *c*. ^{3}H/^{14}C ratios were obtained by Edman degradation of peptides from chymotryptic, thermolytic, and V8-protease digests. Peptides were separated by high-performance liquid chromatography using conditions and equipment as described (Bosshard *et al.*, 1986). Any difference in reaction conditions during labeling of free and bound cytochrome was monitored by the internal standard method, using phenylalanine as an internal standard (Bosshard, 1979; Bosshard *et al.*, 1986).

The reactivity ratio *R* was defined by dividing the ^{3}H/^{14}C ratio of N^6-acetyllysines labeled in free cytochrome *c* by the ^{3}H/^{14}C ratio of the corresponding residues labeled in bound cytochrome *c*. The quantity *R* was a lower estimate of the degree by which the reactivity of a lysine residue was affected through binding to the reaction center; $R > 1$ indicated lower reactivity in bound cytochrome *c*. Based on our past experience, *R* values are in error by 10–15%.

Results

Binding of Cytochrome c and Cytochrome c_2 to the Reaction Center

Binding was measured in a semiquantitative way by gel permeation chromatography (Fig. 1). Complexes were eluted in front of excess unbound cytochrome. The dissociation constant was calculated from the ratio of cytochrome to reaction center in the front peak (Dixon, 1976). The calculation assumed a single cytochrome binding site. Values of K_d obtained in this way were 0.3–0.5 μM for horse cytochrome *c* and 1–1.5 μM for cytochrome c_2 from *R. rubrum*. These values are similar to those reported with the reaction center from *Rps. sphaeroides* (Rosen *et al.*, 1980).

No binding was detected at 200 mM ionic strength (not shown). More notable, however, was the observation that binding of cytochrome c_2 to the reaction center was hardly inhibited by poly-L-lysine. In contrast, no binding of mitochondrial cytochrome *c* to the reaction center was observed in the presence of poly-L-lysine (Fig. 1B).

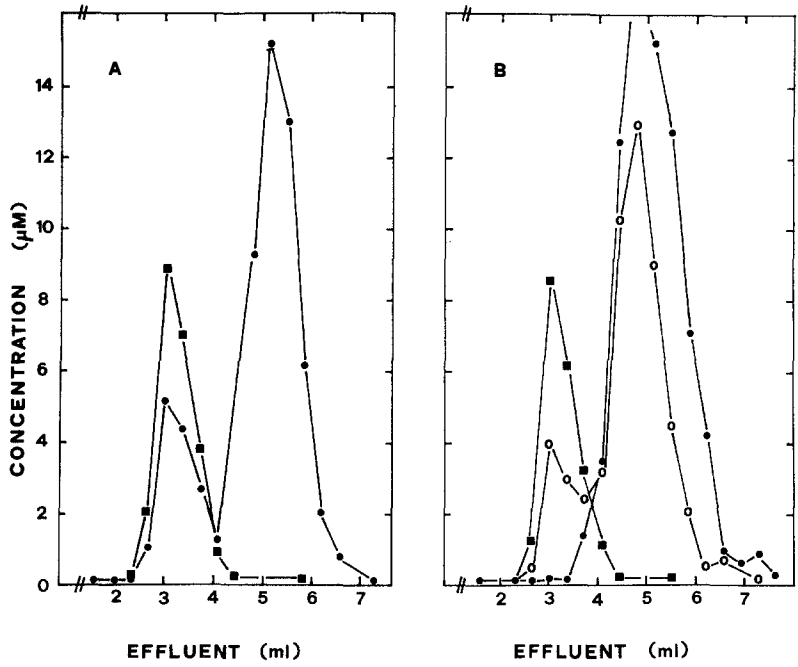

Fig. 1. Binding of cytochromes to the reaction center as measured by gel permeation chromatography. (A) Cytochrome *c* (24.4 μM) and reaction center (8.22 μM) chromatographed on Biogel P100 and eluate analyzed for cytochrome *c* (●) and reaction center (■). (B) Chromatography of cytochrome *c* (42 μM) and reaction center (7.53 μM), and of cytochrome c_2 (26.8 μM) and reaction center (7.65 μM), both in the presence of poly-L-lysine (ca. 6 μM based on average M_r = 22,500). The elution trace of the reaction center is shown only for the experiment with cytochrome *c*. Cytochrome *c* (●), cytochrome c_2 (○), reaction center (■).

Differential Chemical Modification

The principle of the experiment has been described before (Bosshard, 1979; Rieder and Bosshard, 1980; Bosshard *et al.*, 1986). Acetylation of lysines of cytochrome *c* with [^{3}H]acetic anhydride was compared in free and reaction-center-bound cytochrome *c*.

^{3}H-Acetylation of free cytochrome *c* was performed in the presence of the reaction center but at high ionic strength where no binding occurs. The small effect of the ionic strength on the rate of acetylation was corrected for (Rieder *et al.*, 1985). The average number of [^{3}H]acetyl groups per cytochrome *c* was kept below 0.5 mol/mol in order to guarantee that ^{3}H-acetylation *per se* of one lysine does not alter the chemical reactivity of a neighboring lysine residue. By this means, any change in the degree of labeling of a lysine residue was in direct response to the binding event.

Table I. Reactivity Ratio for the Acetylation of Amino Groups of Horse Cytochrome *c* (cyt *c*) in the Presence and Absence of the Photosynthetic Reaction Center from *R. rubrum* (rc)[a]

Residue number			
cyt *c*	cyt c_2	cyt *c*:rc	cyt c_2:rc
	1		3.91
5*		3.20	
7		1.06	
8*	9	3.65	1.26
13*	12	8.00	1.11
	13		1.08
22		0.35	
25*		3.10	
27*	27	3.26	1.25
39		1.10	
	43		0.35
53	56	0.92	0.68
55	58	1.16	0.95
60		0.72	
	72		0.79
72*	75	5.10	1.20
73*		2.05	
	81		0.73
	86		0.71
	88		0.66
79*	90	2.79	0.70
	94		1.30
86*		7.40	
87*	97	4.35	1.58
88		1.40	
99	109	0.80	4.25
100		1.00	
	112		3.90

[a]The results for the cytochrome c_2–reaction center complex (cyt *c*2:rc) are shown for comparison (Rieder *et al.*, 1985). Values underlined differ significantly from unity. Residues at equivalent topographical positions in cytochrome *c* and cytochrome c_2 appear on the same line. Residues marked by an asterisk lay at or close to the binding site of all redox partners tested (Margoliash and Bosshard, 1983).

The degree of labeling of each lysine residue was calculated from $^3H/^{14}C$ ratios (see Experimental). Table I summarizes the reactivity ratios. The photosynthetic reaction center protected lysines 5, 8, 13, 25, 27, 72, 86, and 87 of mitochondrial cytochrome *c*, with residues 13 and 86 being most shielded. The residues protected by the reaction center were, in essence, the same as those found to be protected by mitochondrial reaction partners (Margoliash and Bosshard, 1983; Rieder and Bosshard, 1980). The protected lysine residues are located on the top front of the molecule (Lys 5, 8, 13, 86, 87), to the left (Lys 72), and to the right (Lys 25, 27) of the exposed heme

edge (see Salemme, 1977, for a standard front view of cytochrome *c*). In contrast, those residues of cytochrome c_2 that have been previously identified as being protected in the isolated cytochrome c_2–reaction center complex (Rieder *et al.*, 1985) are on the backside, opposite to the exposed heme edge (N-terminal Glu and Lys 109, 112). Hence, on the basis of differential chemical modification experiments, the reaction center from *R. rubrum* recognizes different surface areas on mitochondrial and bacterial cytochromes *c*.

Discussion

The rate of electron transfer through cytochromes *c* and c_2 is ionic strength dependent, making the mode of binding of the cytochromes to their reaction partners consistent with a plus–minus interaction, with the cytochromes contributing the positive charge (Weber and Tollin, 1985; Margoliash and Bosshard, 1983). As for mitochondrial cytochrome *c*, the present results are in line with this model. The binding domain of mitochondrial cytochrome *c* for the bacterial reaction center is conspicuously similar to the one observed for mitochondrial reaction partners, as far as can be deduced from the results of differential chemical modification (Rieder and Bosshard, 1980). Comparison with our previous data reveals that lysine 27 is slightly more protected and lysines 7 and 8 somewhat less in the cytochrome *c*–reaction center complex than in the complex with cytochrome *c* oxidase and reductase. Incidentially, lysine 22 is *more* reactive in reaction-center-bound cytochrome *c*. This was observed also for cytochrome *c* when bound to the mitochondrial redox partners. This observation points to some subtle yet general conformational effect concomitant to the process of binding.

The present data must be related to our former experiment which indicated that the reaction center could as well bind to the negatively charged backside of cytochrome c_2, i.e., to a site opposite to the postulated electron transfer site on the front of the molecule (Rieder *et al.*, 1985). Since horse cytochrome *c* clearly binds to the reaction center through its positively charged frontside, we must infer that mitochondrial and bacterial cytochromes bind to different sites on the isolated, detergent-solubilized reaction center, even though the sites may overlap. (A ternary complex with both cytochromes could not be isolated by gel chromatography; data not shown.) In line with this conclusion is the present observation that poly-L-lysine inhibits binding of mitochondrial cytochrome *c* much more strongly than binding of cytochrome c_2 (Fig. 1).

One might argue that the lowered reactivity of lysines does not immediately result from direct contact between reaction center and cytochrome. The alternative explanation would be that complex formation induces a

conformational change outside of the intermolecular contact site, thereby rendering some residues less reactive. That frontside residues of mitochondrial cytochrome *c* are shielded directly by several redox partners was amply corroborated by kinetic analysis of singly lysine-substituted cytochrome *c* derivatives (reviewed by Margoliash and Bosshard, 1983; Bosshard *et al.*, 1986). There remains the possibility that in the case of bacterial cytochrome c_2 the reaction center covers the frontside, yet does not alter the chemical reactivity of any frontside residue, changing instead the conformation on the backside of cytochrome c_2 in such a way as to turn three amino groups less reactive toward acetic anhydride. Given the many frontside lysines of cytochrome c_2, the close structural similarity between cytochrome c_2 and cytochrome *c* (Salemme, 1977), and the high thermal and conformational stability of *c*-type cytochromes (Takano and Dickerson, 1980; Dickerson and Timkovich, 1975), this seems to be a very unlikely possibility.

What then is the significance of the backside interaction for the mechanism of electron transfer through cytochrome c_2? We believe that the results with horse cytochrome *c* presented here constitute some indirect evidence against electron transfer through the back of cytochrome c_2 since it is difficult to envisage two different productive electron transfer sites on the reaction center, one positively charged for bacterial cytochrome c_2 and one negatively charged for the unphysiological yet very efficient mitochondrial electron donor. The cytochrome c_2–reaction center analyzed before (Rieder *et al.*, 1985) may not represent a genuine electron transfer complex. Kinetic analysis of the rate of electron transfer with singly lysine-substituted derivatives of cytochrome c_2 may resolve this question. Such derivatives have been used successfully to elucidate the electron transfer domain of mitochondrial cytochrome *c* (Smith *et al.*, 1977; Ferguson-Miller *et al.*, 1978). Incidentally, a stable complex with cytochrome c_2 seems to be no absolute prerequisite for efficient electron transfer (van der Waal and van Grondelle, 1985).

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