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Electrostatic Attraction between Cytochrome bc_1 and Cytochrome *c* Affects Kinetics of Cytochrome *c* Reduction

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Abstract—The kinetics of the ubiquinol-cytochrome *c* reductase reaction was examined using membrane fragments and purified bc_1 complexes derived from a wild-type (WT) and a newly constructed mutant (MUT) strains of *Paracoccus denitrificans*. The cytochrome c_1 of the WT samples possessed an additional stretch of acidic amino acids, which was lacking in the mutant. The reaction was followed with positively charged mitochondrial and negatively charged bacterial cytochromes *c*, and specific activities, apparent k_{cat} values, and first-order rate constant values were compared. These values were distinctly lower for the MUT fractions using mitochondrial cytochrome *c* but differed only slightly with the bacterial species. The MUT preparations were less sensitive to changes of ionic strength of the reaction media and showed pure first-order kinetics with both samples of cytochrome *c*. The reaction of the WT enzyme was first order only with bacterial cytochrome *c* but proceeded with a non-linear profile with mitochondrial cytochrome *c*. The analysis of the reaction pattern revealed a rapid onset of the reaction with a successively declining rate. Experiments performed in the absence of an electron donor indicated that electrostatic attraction could directly participate in cytochrome *c* reduction.

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The membrane-embedded bc_1 complex (Complex III) is a central part of the energy-transducing respiratory chain of mitochondria and many bacteria [1]. The catalytic part of the complex comprises cytochrome *b* (cyt *b*) with two noncovalently attached heme *b* groups (b_L and b_H), the so-called Rieske protein (ISP) with a [2Fe-2S] iron-sulfur cluster, and cytochrome c_1 (cyt c_1) with a covalently attached heme *c* group. The function of the three catalytic subunits is to pass electrons from ubiquinol (QH_2) to cytochrome *c* (cyt *c*) in a quinol-cytochrome *c* reductase reaction (QCR). Depending on the species the bc_1 complexes contain up to eight additional protein subunits. A facultative anaerobic bacterium *Paracoccus denitrificans* possesses a simple cytochrome bc_1 (cyt bc_1) complex consisting just of the three catalytic subunits. A full enzymatic competence of the complex with the sensitivity to specific bc_1 inhibitors has been reported [2]. It has been established that the electron transfer of the QCR reaction is initiated by formation of a transient adduct between the cyt c_1 and cyt *c* [3, 4]. As stated with mitochondrial bc_1 constituents, two types of intermediate adducts could be distinguished in order to transfer electrons to cyt *c*: i) a relatively stable but ionic strength sensitive adduct; ii) an unstable but ionic strength insensitive adduct [5]. This suggests a difference in the binding forces when forming the adducts, a coulombic, electrostatic attraction prevalent when forming the ionic strength sensitive adduct, and a binding force of hydrogen bonding and other mainly hydrophobic attraction giving rise to the ionic strength insensitive adduct. The reaction is then followed by an intracomplex electron transfer from cyt bc_1^{2+} to cyt c^{3+}, and completed by dissociation of the adduct to release the ferrocytochrome c^{2+}. Nowadays it becomes increasingly clear that this simple model of three successive stages is insufficient to include all changes occurring in the process of electron transfer and cannot comply with all experi-

Abbreviations: cyt) cytochrome; MUT) mutant strains of *Paracoccus denitrificans*; QCR) ubiquinol-cytochrome *c* reductase reaction; QH_2) ubiquinol; WT) wild-type strain of *Paracoccus denitrificans*.

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mental data. The information obtained from the residues that have been identified at the interface of the two enzymes indicates that the adduct should be considered as a dynamic ensemble of their different orientations and in equilibrium with the free species in the solution [6, 7]. The crystallization of the cyt bc_1 complex with the bound cyt *c* reveals that the long-range recognition of the interacting components may be driven by nonspecific electrostatic interactions, while at shorter distances hydrophobic contacts are crucial [7, 8]. It is evident that the electron transfer from the cyt bc_1 complex to ferricytochrome *c* may be mediated through cyt *c* experiencing multiple encounters of which only a part may be effective, thus making the kinetic analysis of the reaction difficult [5, 6].

Tian et al. [9], by using the ruthenium–cyt *c* excitation technique, estimated the rate constants of formation of cyt bc_1 and cyt *c* adduct, the rate of intracomplex electron transfer, and the rate constant of dissociation of the product. The values obtained were consistent with an electrostatically assisted diffusion controlled reaction between cyt *c* and the negatively charged cyt c_1. The electron transfer measured in the reverse (cyt c^{2+} to cyt bc_1^{3+}) direction proceeded in two phases: a fast phase proceeding at low ionic strength with first-order rate constants not affected up to ~50 mM NaCl, and a slow phase which obeyed second-order kinetics and sharply decreased with ionic strength increase. The decrease of complex formation brought about by the dissociation of the adduct indicated an involvement of coulombic, electrostatic binding forces [9-11], but their role in the electron transfer between cyt c_1 and cyt *c* remains unclear.

We directed attention to the unique form of cyt c_1 of *P. denitrificans*, which, in contrast to other cyt c_1 species, has an additional sequence of 150 amino acids in the N-terminal domain [12]. The domain situated at the periphery of the molecule is extremely rich in acidic amino acids, namely glutamate residues. It resembles in its composition the separated acidic subunits (QCR8, QCR6) of bovine and yeast bc_1 complexes. We used a new mutant strain of *P. denitrificans* with a truncated copy of cyt c_1 devoid of the stretch of acidic amino acids but possessing the same structural organization of the hydrophobic heme *c* cleft [13]. In the present communication, we compare the effects of varying ionic strength on the QCR reaction of the wild-type (WT) and mutant (MUT) preparations. We analyze the kinetics of the enzyme reaction using various concentrations of positively charged mitochondrial cyt *c* and negatively charged bacterial cyt *c*. The results obtained confirm a participation of electrostatic attraction in the QCR reaction.

MATERIALS AND METHODS

Materials and reagents. Cytochrome *c* from horse heart, type VI, was purchased from Sigma (USA). The bacterial cyt *c*, soluble, with molecular mass 14,287, from *P. denitrificans* (cyt $c_{P.d.}$) was separated and purified to electrophoretic homogeneity as described [14]. 2,3-Dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone (Q_2) was kindly donated by F. Rohl (BASF, Germany). The concentration of Q_2H_2 was determined using ε_{red-ox} = 12.5 $mM^{-1}\cdot cm^{-1}$ at 275 nm [15]. Antimycin A was from Sigma and applied in ethanolic solution using ε = 4.8 $mM^{-1}\cdot cm^{-1}$ at 320 nm [16].

Generation of *P. denitrificans* strain. For expressing a functional cyt bc_1 complex with a truncated cyt c_1 the coding region of the *fbcC* gene between triplet 39 and 202, specifying the complete highly acidic domain of the *P. denitrificans* cyt c_1, was deleted in-frame by restriction digest with XhoI and NotI (removing nucleotides 2279 to 2766) followed by blunt-end generation and re-ligation. The resulting plasmid pEG471 was transferred into strain G440 yielding a G440/pEG471 strain (MUT), as detailed in [13]. As a wild-type (WT) strain, *P. denitrificans* ATCC 13543 was used.

Enzyme preparations and activity assay. The strains were grown aerobically on succinate, and membrane fragments were obtained by mechanical disintegration and differential centrifugation as described [17]. Protein concentration was determined by the method of Lowry. Quantifications of the content of cyt c_1 in membrane fractions were performed immunologically by Western blotting, using specific antibodies and alkaline phosphatase-linked protein A, as described [13]. The QCR activity of membrane fragments was assayed spectrophotometrically in 1 ml of reaction mixture by measuring the reduction of cyt *c* by Q_2H_2. The potassium phosphate buffer, pH 7.3, contained 1 mM EDTA, 1 mM KCN, 20 or 50 μM Q_2H_2, and 2.9 or 3.1 μg of membrane protein of the MUT- and WT-strains, respectively, i.e. 0.38 nM MUT-bc_1 or 0.4 nM WT-bc_1; the reaction was started by addition of cyt *c*. The enzyme activity was determined from the increase of absorbance at 550 nm using millimolar extinction coefficients ε = 27.6 and 26.8 $mM^{-1}\cdot cm^{-1}$ for the reduced cyt *c* of horse heart and *P. denitrificans*, respectively, and expressed in units (U) defined as 1 μmol of cyt *c* reduced per min per mg protein and in turnover numbers (TN) per mol of cyt c_1 given also as apparent k_{cat} under nonsaturating substrate conditions. In estimating the difference of absorption between reduced and oxidized form of cyt *c*, $\Delta\varepsilon_{550}$ = 19 $mM^{-1}\cdot cm^{-1}$ was used for both (mitochondrial and bacterial) samples. The nonenzymatic reduction of cyt *c* by Q_2H_2 was determined separately and subtracted. The cyt bc_1 complex was purified from the dodecyl maltoside extract of membrane fractions according to [18]. The contents of hemes were determined using $\Delta\varepsilon_{560-574}$ = 28 $mM^{-1}\cdot cm^{-1}$ and $\Delta\varepsilon_{553-540}$ = 17.1 $mM^{-1}\cdot cm^{-1}$ for heme *b* and c_1, respectively. Both samples were diluted with an equal volume of glycerol and stored at −80°C. The QCR activity of the purified bc_1 complexes was measured with 20 ng of protein (0.14 and

0.17 nM of heme c_1 for WT and MUT samples, respectively) in a separate medium of 50 mM potassium phosphate buffer, pH 7.3, containing 0.2 g/liter of dodecyl maltoside. Samples of the stock solutions of the bc_1 complexes were suspended before use in a dilution buffer (50 mM potassium/sodium phosphate, pH 7.3, 0.2 mM NaCl, 0.5 g/liter dodecyl maltoside, 1 mM EDTA, and 10% of glycerol) to a concentration of 10 μg protein per ml. The dilution was made freshly for each activity assay and the sample used within 2 h.

Kinetic analysis of the QCR reaction. The K_m values for cyt *c* were determined from initial rates of the QCR reaction using 0.4 up to 20 μM concentrations. Steady-state kinetics of the QCR reaction was studied under conditions when the progress of cyt *c* reduction showed both zero-order and a first-order region; at lower cyt *c* concentrations, the kinetics of the reaction could be followed as first-order [19]. The kinetic responses for the first order rate constants were calculated from a non-linear least-squares analysis according to the equation

$$A = b_0 + b_1\exp(b_2 \cdot t) \quad (1)$$

with the aid of a computer fitting program written in Matlab. The use of the equation made possible an accurate determination of the values of the constants in arbitrary selected time intervals. In a graphical presentation of the equation, *A* (absorbance) are values plotted on the *y* axis, the data for which were collected from an increase in 550 nm absorption at 0.01 min intervals in time periods lasting up to 2 min, b_0 means the zero shift of the absorbance values (A_0), b_1 is the multiplication factor according to the time scale, and b_2 is a slope of the graphical presentation expressing the first order rate constant. Because the real QCR reaction is more complex than a single variable case, we prefer to call the estimated kinetics as pseudo-first order. Calculations for the second order reaction were performed according to the equation:

$$1/(A_t - A_0) = 1/A_0 + 1/k \cdot t, \quad (2)$$

where A_t and A_0 are absorbance values at time *t* and time zero. The typical best fit curves obtained from the rate constants multiplied by the cyt *c* concentration are shown in Fig. 1 (experimental model). It can be seen that calculations for the first- and second-order reactions gave values that were very similar in the time intervals of the experiments; the description of the reaction may be arbitrary in some experiments. Divergences in the rate observed throughout the process of the reaction were analyzed by means of the graphical presentation of the log intensity versus time yielding a strait line in the case of the first order reaction (logarithmic model).

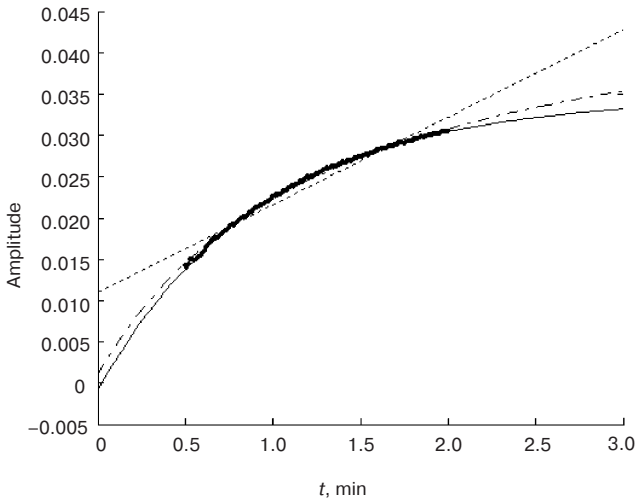

Fig. 1. Graphical presentation of ubiquinol-cytochrome *c* reductase (QCR) activity according to the reaction order. Heavy dots are experimental data, lines (---), (——), and (– · –) mean values calculated according to the zero, first, and second orders, respectively.

RESULTS

Characteristics of enzymic preparations. The site-directed mutagenesis of cyt c_1 to its truncated copy resulted in a change in the amino acid content. The 102 alanine residues of the original cyt c_1 were decreased to 40 residues, the 26 aspartic acid residues were decreased to 18, and those of glutamic acid from 70 to 18. The ratio of the absorption maxima at 560 minus 574 nm to 553 minus 540 nm was close to one (1.09 and 1.05) for the WT and MUT membrane protein, respectively, and increased to 1.72 and 1.67 in purified WT and MUT bc_1 preparations (Table 1). This indicates that the WT and MUT enzymic fractions are comparable with respect to the contents of cyt *b* and cyt c_1. Although the analysis of the WT and MUT membrane fractions showed different contents of cyt c_1 protein, they did not differ in its molar amount when calculated on the basis of molecular masses for the wild-type enzyme (46,845) and for the truncated cyt c_1 molecule (30,599). There was only a slight decrease (10-15%) in the QCR activity given in units and apparent k_{cat} values with the MUT protein. This indicates that the binding sites for cyt *c* on the MUT cyt c_1 were not impaired. The K_m values for cyt *c* calculated for the QCR reaction yielded values of 5.5 ± 0.8 and 7.14 ± 0.6 μM for the WT and MUT membrane protein, respectively. At concentrations of cyt *c* higher than 15 μM the rates of QCR activity was almost unchanged, indicating that reactions other than the electron transfer between the cyt c_1 and cyt *c* become rate limiting [19, 20]. A profound increase in the rates of QCR activities was obtained with purified bc_1 complexes. This finding is in keeping with a published observation [21]. In this case, we detected a significant lowering of the QCR activity at the MUT bc_1

Table 1. Characteristics of enzymic proteins derived from *Paracoccus denitrificans* strains

Enzymic fraction	Cytochrome c_1		$A_{560\text{-}574}/A_{553\text{-}540}$ ratio	QCR activity[a,b]	
	μg/mg	nmol/mg		U/mg	k_{cat} (sec^{-1})
Membranes					
wild-type	6.2	0.13	1.09	1.35	170
mutant	4.0	0.13	1.05	1.29	165
Complexes bc_1					
wild-type	—	7.1	1.72	469	919
mutant	—	8.3	1.67	308	517

[a] Relative standard deviation taken from five repetitions using reaction mixture (see "Materials and Methods") with 50 mM phosphate buffer and 10 μM cyt *c* was 9% for membrane fractions. For the variance of enzymic activity of purified bc_1 complexes, see text.

[b] Due to different sets of membrane preparations and a different composition of the reaction medium for the bc_1 complexes, the activities in the upper half of the table are not strictly comparable to the lower half.

complex in comparison with that of the WT preparation as shown in Table 1. The apparent k_{cat} values calculated for the WT sample ranged between 711 and 1048 sec^{-1} and between 467 and 664 sec^{-1} for the MUT bc_1 complex. The V_{max} of the MUT bc_1 complex decreased approximately to half of that of the WT sample, and the K_m values for cyt *c* in the MUT bc_1 complex increased two to three times (not shown).

Sensitivity to specific inhibitors. A good measure for native assembly and electron flow through catalytic subunits is the sensitivity to specific respiratory inhibitors of the bc_1 complex. We tested the QCR activity of WT and MUT fractions to myxothiazol, MOA-stilben, stigmatellin (not shown), and performed in detail the inhibitory analysis with antimycin. The effect of antimycin on the WT membrane fraction measured with 1 μM cyt *c* showed a steep rise in the inhibitory effect between 0.1 and 0.5 nmol antimycin/mg protein. The inhibition of antimycin with the MUT fraction maintained a similar pattern with slight differences at lower and higher antimycin concentrations (Fig. 2). In the 0.01 to ~0.1 nmol range of antimycin/mg membrane protein, a stimulation of enzymic activity up to ~20% with the two samples was observed. Both preparations showed a similar inhibitory profile to increasing concentrations of antimycin in which the effect gradually switched to inhibitory action. The specific activities found with 0.028, 0.054, and 0.078 nmol of antimycin per mg MUT protein were 145, 135, and 106 mU, respectively, in comparison to 128 mU found with the control sample (for the standard deviations see Fig. 2). At high concentrations of antimycin (1-5 nmol/mg protein), the MUT enzyme was almost completely inhibited, whereas the WT enzyme retained about 20% of its activity. From the molar content of cyt c_1 it follows that the steep rise in the antimycin effect on WT as well as MUT samples takes place between 1 and 5 nmol antimycin/nmol cyt c_1 that is at about 1 : 1 antimycin/cyt *b* ratio. The value is, with respect to our experimental conditions, comparable to values found with preparations from other bacterial sources. The QCR reaction measured with membrane fragments using the limiting (1 μM) cyt *c* concentration provides a similar inhibitory pattern as do the purified bc_1 complexes measured in the presence of 5 μM concentration of cyt *c* (not shown). The results indicate that the electron flow through the modified bc_1 complex remains unperturbed and exhibits characteristic features of that observed with the WT preparations.

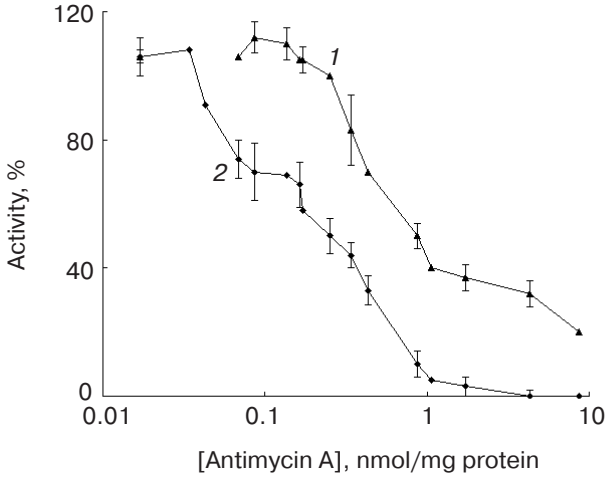

Fig. 2. Activity of the QCR reaction in the presence of antimycin. Membrane fragments of *Paracoccus denitrificans*, 3.1 μg protein wild-type (*1*), 5.8 μg protein mutant (*2*), in 50 mM phosphate buffer mixture; the reaction was started by addition of 1 μM mitochondrial cyt *c*. Values given with error bar are the mean of triplicate.

Effect of ionic strength. The effect of increasing the ionic strength on the activities of the QCR reaction for the WT and MUT fractions was followed in reaction mixtures under increasing cyt *c* concentration. The highest values of activity of the two preparations were obtained in media of low ionic strength that was in 5 mM (not shown) and 10 mM phosphate buffer (Table 2). There was a difference in the QCR activity between the WT and MUT membrane protein that was clearly observed when the cyt *c* concentration was low, i.e. when the reaction between cyt *c* and cyt c_1 became rate-limiting [19]. Table 2 also shows the TN values related to the molar content of cyt c_1. A similar response of the values given in units and turnover numbers to the change of the ionic strength indicates that the truncated copy of cyt c_1 in the MUT fraction is equally accessible to the reaction with cyt *c* similarly to what has been found with the WT preparation. The relative decrease in the enzyme activity of the MUT sample in comparison to the WT preparation was most pronounced at 0.5 and 1 μM cyt *c* concentrations. In medium of 10 mM buffer, the difference in enzyme activities between WT and MUT membranes at these low cyt *c* concentrations was about 40%, whereas only about 10% decrease was detected with 10 μM cyt *c*. There was

Table 2. Effect of ionic strength on the QCR activity of *Paracoccus denitrificans* membranes[a]

Membrane fragments	Phosphate buffer (pH 7.3) concentration, mM	Cytochrome *c*, μM							
		0.5		1		5		10	
		mU	TN, sec^{-1}	mU	TN, sec^{-1}	mU	TN, sec^{-1}	mU	TN, sec^{-1}
Wild-type	10	507	65	988	126	1247	159	1442	183
	50	333	43	520	66	920	117	1353	172
	100	130	17	326	42	780	99	1247	159
	250	<20	<2.5	110	14	210	26	858	110
Mutant	10	312	40	511	65	851	109	1414	180
	50	207	26	426	54	723	92	1277	163
	100	114	15	298	38	681	87	1234	157
	250	<20	<2.5	170	22	281	36	979	125

[a] A single batch of enzyme was used for each concentration of the buffer. The mU is defined as 1 nmol of cyt *c* reduced per mg protein per minute. The RSD within a single preparation batch was ± 6% (±11 sec^{-1}/180 sec^{-1}).

Table 3. First order rate constants of the QCR reaction with rate-limiting concentrations of cyt *c*[a,b]

Membrane fragments	Phosphate buffer (pH 7.3) concentration, mM	Cytochrome *c*, μM					
		0.5		1		5	
		a	b	a	b	a	b
		k (sec^{-1})					
Wild-type	10	45.5	7.6	31.7	7.3	12.8	3.5
	50	20.3	6.6	12.2	6.0	6.7	2.5
	100	7.9	2.6	5.6	4.4	4.7	2.0
Mutant	10	29.7	6.9	14.1	6.2	6.8	3.4
	50	17.4	6.6	8.8	5.9	5.2	2.2
	100	8.6	1.4	6.4	2.9	4.5	1.8

[a, b] The values were obtained with cyt *c* (mitochondrial cyt *c*) and cyt $c_{P.d.}$ (bacterial cyt *c*), respectively. The relative standard errors of estimates varied between 5.8-15.5%.

also a distinct response of the QCR activity between WT and MUT fractions to the increase in ionic strength, showing a lower sensitivity of the latter. A drop in activities to 28, 33, 62, and 85 ± 6% with 0.5, 1, 5, and 10 μM cyt *c*, respectively, was found with WT particles in 100 mM buffer in comparison to the activity found in 10 mM buffer (100%). The activity of MUT fraction was less affected by changing the concentrations of the buffer, i.e. 37, 58, 80, and 90 ± 6%; at 250 mM buffer concentration the activity of the MUT sample exceeded the activity of the WT sample (cf. Table 2). Control experiments in which part of the potassium phosphate was substituted by potassium chloride without changing the ionic strength of the medium did not affect the data (not shown), confirming that the observed change in activity was due to ionic strength effect. The results show that the suppression of electrostatic attraction by increasing the ionic strength affects more markedly the QCR activity of the WT preparation. These findings point to participation of electrostatic attraction in the electron transfer between cyt *c* and the cyt c_1.

The distinguishable effect of ionic strength on the QCR activity of the WT and MUT membranes was further confirmed by estimating the first-order rate constants k (sec^{-1}) with low concentrations of the two cyt *c* as given in Table 3. There was a clear difference in the k (sec^{-1}) values between the WT and MUT particles using cyt *c* in reaction media containing 10 mM buffer and practically no difference in medium of 100 mM buffer. In 50 mM buffer media, the first-order rate constants were generally much higher at low concentrations of cyt *c* (0.5 and 1 μM); with 5 μM concentration they decreased markedly. There was also a corresponding difference of the first-order rate constant values in the sensitivity to ionic strength between the two preparations. The increase in the first-order rate constants observed between 100 and 10 mM buffer with 0.5 and 1 μM cyt *c* was about 6-fold with WT particles, and the rise in the k (sec^{-1}) values with MUT particles was about 3-fold. It is evident that the interaction between the native cyt c_1 possessing a stretch of acidic amino acids responds more sensitively to the change in ionic strength than does the truncated copy of cyt c_1 in the MUT strain. Table 3 also shows the k (sec^{-1}) values of the two preparations obtained with the cyt $c_{P.d.}$. This constitutive soluble cyt *c* of *P. denitrificans* is acidic (pI = 4.6) due to the total content of 19% acidic and only 14% basic amino acids. However, the structural organization of the heme *c* cleft and the redox potential are identical with mitochondrial cyt *c*. The experiments with cyt $c_{P.d.}$, used as an electron acceptor in the QCR reaction, revealed only a small difference (up to 15%) in specific activities (not shown) and k (sec^{-1}) values between the WT and MUT membranes. These values were much less dependent on increasing the ionic strength of the media and not so clearly affected by increasing the cyt $c_{P.d.}$ concentration (cf. Table 3). The results obtained with cyt $c_{P.d.}$ show that acidic residues present on its molecule markedly lower the kinetic parameters of the QCR reaction. The conforming enzymic activity of the WT and MUT fractions with the acidic cyt $c_{P.d.}$ and the less pronounced dependence on ionic strength are in keeping with the varying involvement of electrostatic attraction in the process of electron transfer between cyt c_1 and cyt *c* species.

Kinetic analysis of the QCR reaction. The kinetic evaluation of the QCR reaction was performed with low cyt *c* concentrations at which the difference in activities between the used preparations was the maximum. The curves of cyt *c* and cyt $c_{P.d.}$ reduction for membrane fragments of the WT and MUT samples are given in Fig. 3 (a and b). The experimental points of measurement of the 550 nm absorption obtained at low ionic strength (5 mM phosphate buffer) and at 1 μM cyt *c* concentration are overlaid with the line calculated according to the given equation for the first-order reaction. It can be seen that the reaction, performed with bacterial cyt $c_{P.d.}$, exhibits a regular first-order reaction rate with both WT and MUT samples. Both samples show similar values of first-order rate constants, i.e. 1.047 and 0.714 min^{-1} for the WT and MUT fragments, respectively. These reaction rates of cyt $c_{P.d.}$ reduction are stable over the whole time interval of measurement, as confirmed by a logarithmic graphical presentation given in Fig. 3b. On the other hand, the reaction profiles obtained using mitochondrial cyt *c* show non-linear behavior. The reaction starts with a rapid phase of cyt *c* reduction, which is more active for the WT sample and does not proceed at the same rate to complete the cyt *c* reduction; the rates slow transiently and increase again. A significant decrease in the rate of the cyt *c* reduction could be seen with WT and to some extent also with MUT samples at a later stage of the reaction, i.e. when more than 50% of the cyt *c* was reduced (cf. Fig. 3b). A similar decrease in the rate of cyt *c* reduction in the QCR reaction was already observed and analyzed under single turnover conditions of the purified bc_1 complex [21, 22]. At the later stage of the reaction, also the well-known product inhibition from oxidized cyt *c* may be involved. We focused our attention on the onset of cyt *c* reduction, which means to the ~30 sec initial interval through which the reaction rate seemed to be linear. The first-order rate constants for this interval calculated from the curves according to the given equation were 2.878 min^{-1} with the MUT and 6.195 min^{-1} with the WT samples. The graphical logarithmic presentation of this interval at the WT sample revealed a reaction pattern with a successively declining reaction rate, unlike the linear rate obtained with the MUT sample (Fig. 4, a and b). The divergence from the first-order reaction rate could be largely suppressed on increasing the ionic strength and at increased concentrations of cyt *c* in the reaction medium. This may be seen on the curves obtained with 5 μM cyt *c* concentration in 10, 50, 100, and 250 mM buffer (Fig. 5). The

difference in kinetic characteristics for the cyt *c* reduction observed with the WT and MUT membrane fragments was also found with the purified bc_1 complexes of the two strains. Figure 6 (a and b) presents experimental and logarithmic curves of cyt *c* reduction obtained with the bc_1 complex of the MUT strain. There are only slight differences in the shape of the curves caused probably by a nonenzymatic transfer of electrons from Q_2H_2 to cyt *c* on changing the sequence of additions into the reaction mixture. Nevertheless, it is evident that the reduction proceeds to its completion at a stable reaction rate consistent with the first order. Unlike that, the reaction curve obtained with the purified bc_1 complex of the WT strain revealed a successive decline in the reaction rate from its rapid beginning similarly to that observed with membrane fragments (cf. Fig. 7, a and b). The same course of the reaction was also found in the presence of myxothiazol, indicating that the decline cannot be due to the difference in reaction rates between the WT and MUT enzymes. The unusual reaction pattern consistent neither with first-order nor second-order reactions invokes the idea of the involvement of an additional mechanism of cyt *c* reduc-

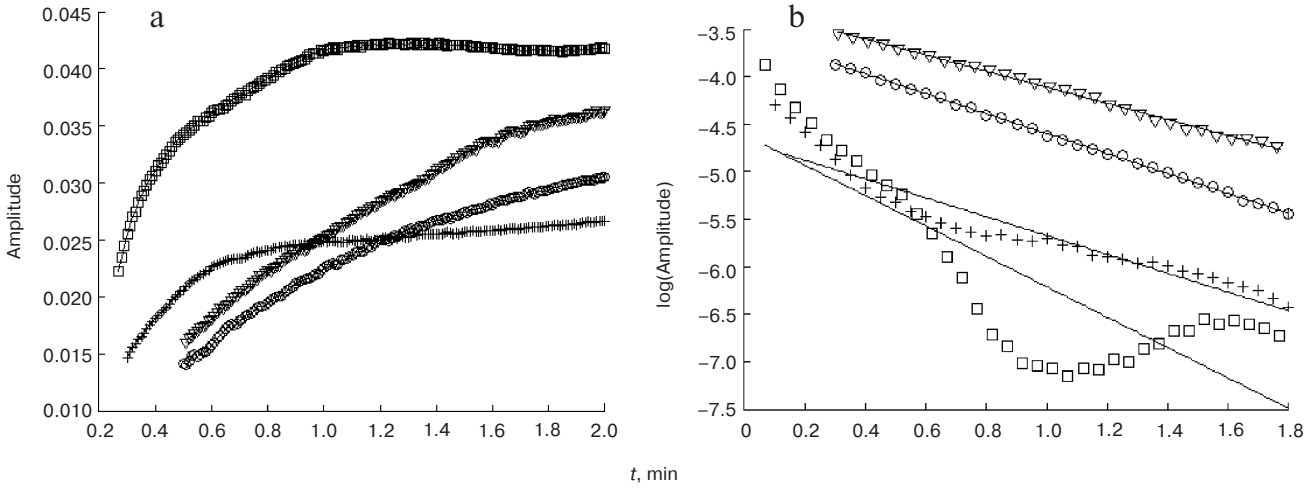

Fig. 3. Reduction curves in the QCR reaction with membrane fragments of *Paracoccus denitrificans*. Experimental data obtained with 3.1 μg protein (0.4 nM bc_1), WT (squares, circles), and 2.9 μg protein (0.38 nM bc_1), MUT (crosses, triangles), are overlaid with the line calculated according to the first order. The reaction was started by addition of 1 μM bacterial cyt $c_{P.d.}$ (triangles, circles), or by addition of 1 μM mitochondrial cyt *c* (squares, crosses). a) Experimental model; b) logarithmic model (only one of three successive points is presented).

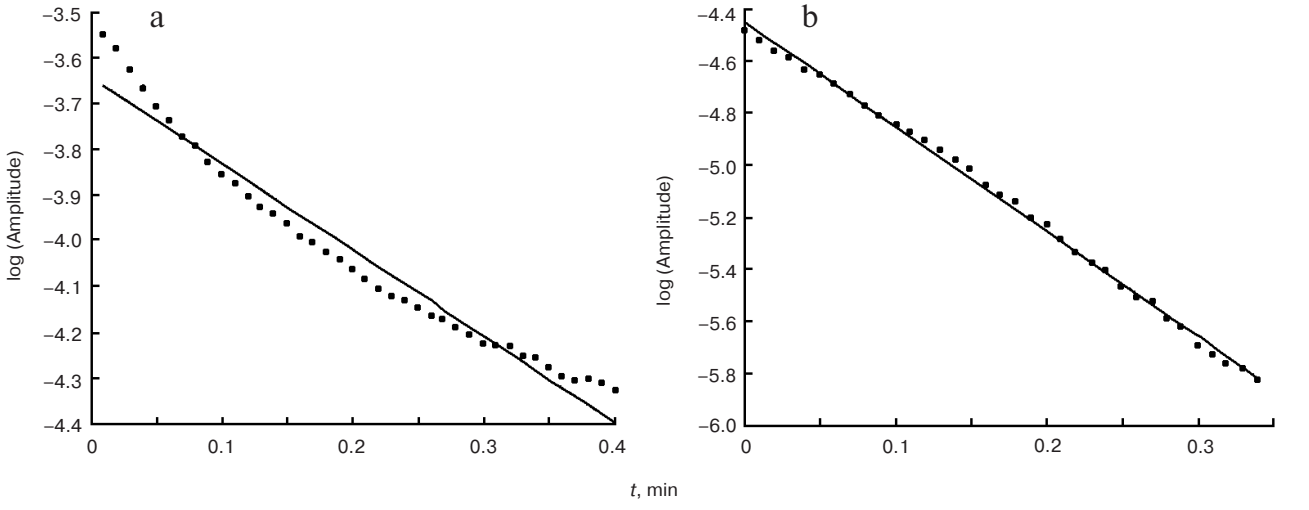

Fig. 4. Evaluation of the first intervals of the QCR reaction. Logarithmic model. Measurement using mitochondrial cyt *c* (dots) with the WT membrane protein (a) and with the MUT membrane protein (b). The parameters of the calculation (line) were: a) $A_0 = 0.043$; $b_1 = 0.08238$; $b_2 = 6.195$; RSS = 1.0433exp^{-6}, $r = 0.99929$; b) $A_0 = 0.05$; $b_1 = 0.032237$; $b_2 = 1.4603$; RSS = 2.0208exp^{-7}; $r = 0.99859$. The composition of the reaction mixture was as given in Fig. 3.

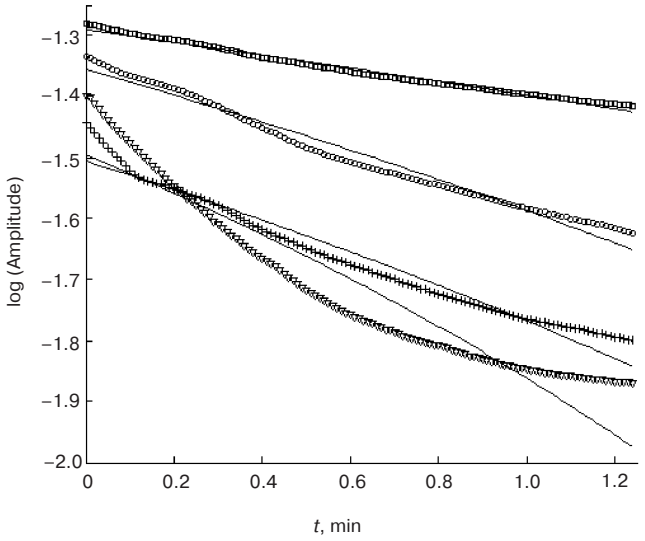

Fig. 5. Effect of ionic strength on the reduction of cyt *c* measured with the WT membrane fraction. Logarithmic model. The mixtures were in phosphate buffer, pH 7.3: 10 mM (triangles), 50 mM (crosses), 100 mM (unfilled circles), 250 mM (squares); with 3.1 μg protein (0.4 nM bc_1). The reaction proceeded with 5 μM mitochondrial cyt *c*.

tion. The finding that the deviation from the first order detected with WT preparations does not take place with equally charged (acidic) cyt $c_{P.d.}$ suggests a role of electrostatic interaction in some additional process participating in cyt *c* reduction. This idea has been supported by experiments performed with MUT preparations lacking the negative charge essential for electrostatic attraction. As shown above (cf. Figs. 4 and 6b), the QCR reaction of membrane fragments and the bc_1 complex derived from the MUT strain proceeded close to the first order with mitochondrial as well as with bacterial samples of cyt *c*.

Involvement of intermediate reactions. To examine the idea of an additional process leading to cyt *c* reduction, control experiments were performed with 0.5 and 1 μM cyt *c* using a 5 mM phosphate buffer and a complete reaction mixture but lacking membrane particles and with no electron donor. No cyt *c* reduction was observed. In a further experiment, these low concentrations of cyt *c* were injected into a mixture containing WT membranes in an amount yielding a 0.4 nM cyt bc_1 concentration but no electron donor. In this case a burst in 550 nm absorption was observed (Fig. 8) indicating a change in the redox state of cyt *c*, which slowly disappeared over a ~15 sec interval. In the first part of the interval a slow absorbance increase at 550 nm with apparent oscillations was observed reaching a maximum which would be obtained when about two thirds (69-72%) of cyt *c* present in the reaction mixture were converted to the reduced form. A subsequent decrease of absorption to the original value indicates a redox transition in the reverse direction. The next addition of 1 μM cyt *c* into the mixture showed a similar but slower response in the increase of 550 nm absorbance. This change in absorption should be observed if 16 ± 5% of the total amount of cyt *c* in the mixture were reduced. The absorbance increase was also evident on further cyt *c* additions, but it was absent in media of ionic strengths of the buffer higher than 50 mM (not shown). No such burst of 550 nm absorption could be observed with the MUT membranes, indicating that the change in absorbance could not be caused by any change in physical state of the particles. The experiments were also repeated with a soluble, purified WT-bc_1 complex in a detergent-containing mixture (see "Materials and Methods" section). A similar absorption change indicating a partial reduction of cyt *c* without the input of an external electron donor was observed. These experiments were performed in an opposite way, by injecting

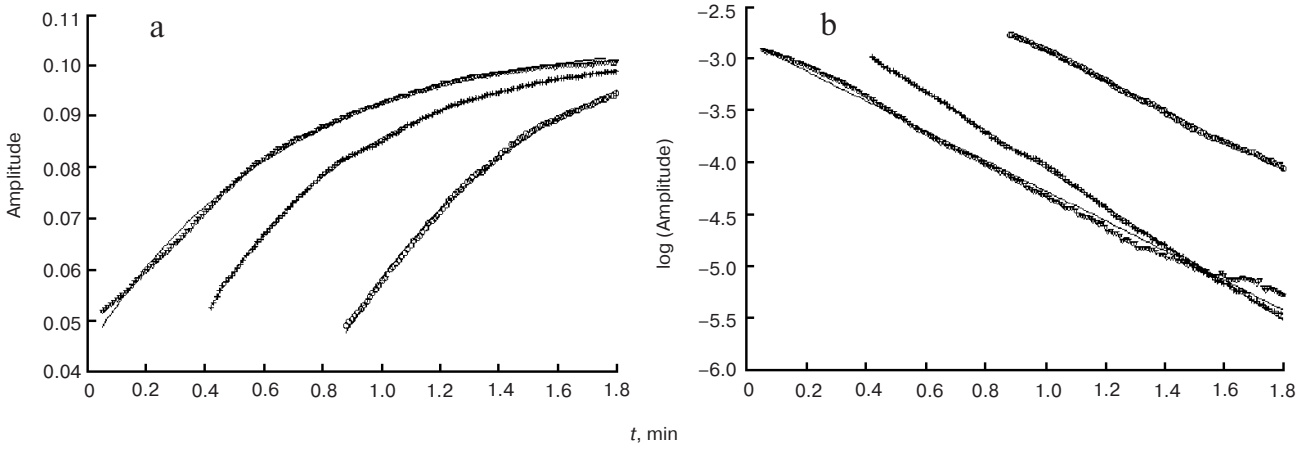

Fig. 6. Reduction profile of cyt *c* measured with purified MUT-bc_1 complex. The medium was in 50 mM phosphate buffer, pH 7.3, containing 0.2 g/liter of dodecyl maltoside, 5 μM cyt *c*; the reaction was started by addition of 50 μM Q_2H_2 (triangles, unfilled circles) or by 0.17 nM bc_1 (crosses); the values calculated according to the first order are depicted as lines. a) Experimental model; b) logarithmic model.

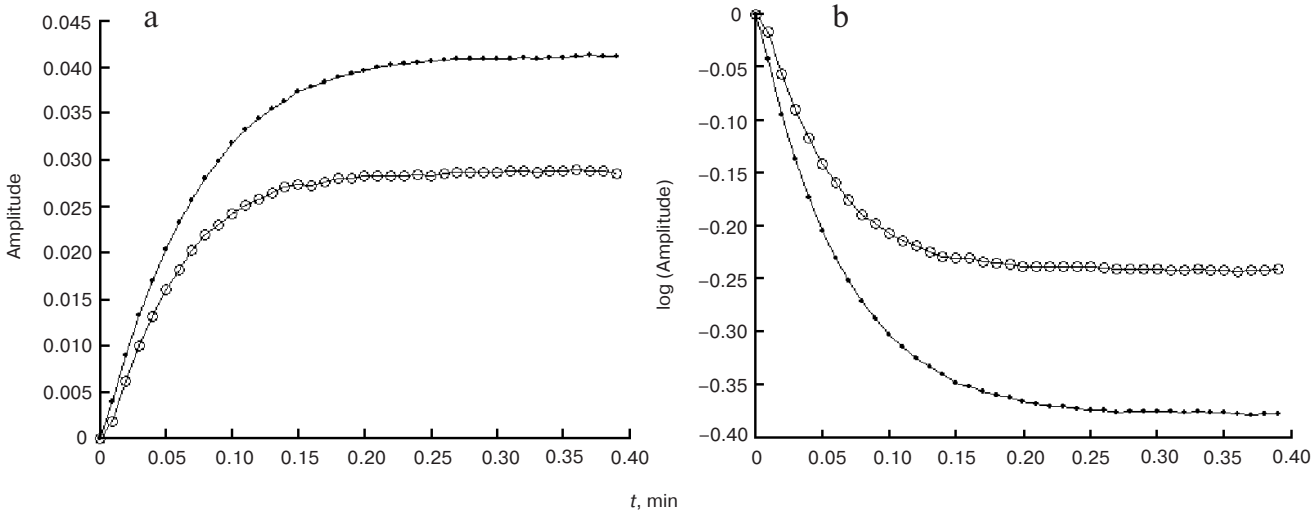

Fig. 7. Reduction profile of cyt *c* measured with purified WT-bc_1 complex. The medium was as stated for Fig. 6; the reaction was started by 0.14 nM of bc_1. Measured values (dots); in the presence of 0.05 nmol of myxothiazol (empty circles). a) Experimental model; b) logarithmic model.

5 µl of the complex (0.35 nM bc_1) into a reaction mixture containing 1.5 µM cyt *c*. In Fig. 9, we show the response of 550 nm absorption to three successive additions of 0.35 nM bc_1 performed in this case in medium of 5 mM Tris-HCl, pH 7.3. The increments of 0.35 nM bc_1 brought about a reversible absorption change equivalent to a conversion of 17 down to ~10% of cyt *c* in ~30-40 sec time intervals. The results show that also with soluble enzymes the idea of the cyt *c* reduction driven by an electrostatic process is acceptable. The interpretation is strengthened by the fact that also in these experiments no change in absorption was observed with the MUT-bc_1 preparation. The identical observation made with the purified enzymes also excludes a possibility that the effect might be due to a dynamic manner of opening and closing the membrane vesicles. We are inclined to conclude that this reaction may be responsible for the observed divergence from the linear reaction rate; structural changes on the proteins leading to cyt *c* reduction may apparently contribute to the forward electron transfer from cyt c_1 to cyt *c*.

DISCUSSION

The present experiments have been devoted to the long discussed question of electrostatic attraction in the

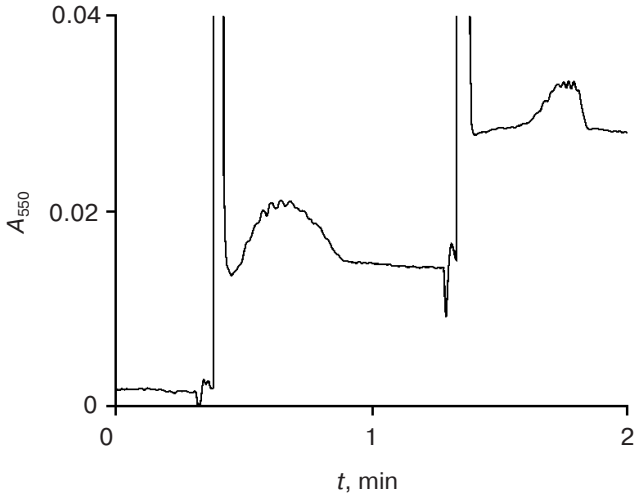

Fig. 8. Transient change of 550 nm absorption in a reaction mixture without an electron donor. To the reaction mixture of 5 mM phosphate buffer, pH 7.3, containing 3.1 µg of the WT-membrane preparation, 0.5 and 1 µM of cyt *c* was added as indicated by ǁ.

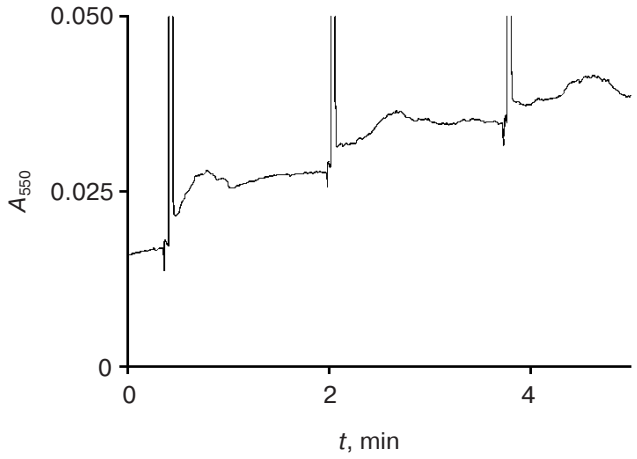

Fig. 9. Reversible change in 550 nm absorption on addition of WT-bc_1 to cyt *c* solution. To the reaction mixture of 5 mM Tris-HCl buffer, pH 7.3, containing 1.5 µM cyt *c*, 35 nmol of the bc_1 complex was successively injected as indicated by ǁ.

process of electron transfer between cyt c_1 and cyt *c*. To address the question a unique form of cyt c_1 as found in the bc_1 complex of the wild-type strain of bacterium *P. denitrificans* was chosen and subjected to site-directed mutagenesis. A mutant strain possessing a truncated copy of cyt c_1 was constructed in which the stretch of 150 amino acids present in the N-terminal domain of the wild-type protein was removed. The domain is extremely rich in acidic amino acids closely resembling in its structure the separated subunits of bovine and yeast bc_1 complexes (QCR8 and QCR6). We anticipated that our approach to examine the role of electrostatic attraction would be less deleterious to the native structure of the bc_1 complex than were previous experiments addressing the question in which mitochondrial bc_1 complexes devoid of the acidic subunits were used. These preparations were less stable showing changes in physical parameters and in the heme *b* to heme *c* ratio [16, 23, 24]. The characteristics of our preparations derived from the MUT strain shows that there was no marked loss of the two hemes caused by mutation, and a similar heme *b*/heme *c* ratio was found in membrane as well as in the purified bc_1 protein. The marked increase of the heme *b*/heme *c* ratio observed in purified bc_1 proteins of both strains was obviously due to the removal of additional membrane-bound *c*-type cytochromes, namely cyt c_{552}. This serves as a mediator of electron transport from cyt c_1 to oxygen and was found not to interfere in the reductase reaction with soluble cytochromes *c* [25]. Nevertheless, all QCR experiments with membrane fragments were performed under conditions under which the electron transfer to cytochrome oxidase was inhibited. The increase in the apparent k_{cat} values observed with purified bc_1 complexes may be ascribed to the effect of detergent in the purification procedure and the presence of detergent and glycerol in the reaction media used [18]. We performed our measurement also in detergent-free media using unperturbed membrane fractions, for preserving integrity of enzymic protein in its native phospholipid surrounding. A reduced ubiquinone homolog (Q_2H_2) was chosen to provide electrons for transfer to cyt *c* in the QCR reaction. Our preference for Q_2H_2 was its high catalytic center activity, which easily made conditions of the QCR reaction rate-limiting for cyt *c*.

The QCR activity of the WT and MUT membrane samples exhibited similar rates of activity of the cyt *c* reduction when measured in zero-order region under cyt *c* saturation. However, under these conditions, the electron transfer is limited by the movement of the protein domain in the ISP cluster (Rieske iron-sulfur protein), which has to move from the "fixed" position to the "released" position [20], and the kinetics of the electron transfer between cyt c_1 and cyt *c* cannot be exactly determined. Calculations for the first- and second-order reactions gave values that were very similar in the investigated time interval (cf. Fig. 1). It was evident that the inclusion of less or more experimental points could affect the decision about the reaction order. This was proven by testing the confidence intervals of correlation coefficients when measured in time periods of 1, 1.5, and 2 min (data not shown). The other observation, i.e. lowering of the first-order rate constant values on increasing cyt *c* concentration, has been known for a long time [19]. Our experiments performed with low concentrations of cyt *c* revealed a substantial decrease of the QCR activity at the MUT samples in comparison with the WT preparations. The difference in apparent k_{cat} and first-order rate constant values was greatest in media of low ionic strength, i.e. under conditions of maximal involvement of electrostatic attraction. Accordingly, the impairment of coulombic interaction due to the absence of negative charge on the molecule of MUT cyt c_1 was manifested in a lower sensitivity to ionic strength of the samples. There was also a substantial decrease in the QCR activity on using acidic cyt $c_{P.d.}$, and a linear profile of the reaction was obtained both with the WT and MUT preparations. Unlike that, an unusual course of reaction could be seen with the oppositely charged mitochondrial cyt *c* in the WT samples. The attenuation of reaction rates observed with cyt *c* at the later stage of the reaction might be partly ascribed to the fact that the reaction rate with mitochondrial cyt *c* was higher and the depletion of substrate faster compared to cyt $c_{P.d.}$. Our attention was focused upon the gradual decline in the reaction rate observed just after the start of the reaction. The behavior may consist in formation of adducts between cyt *c* and its redox partner, which is associated with a conformational change in the active sites of both proteins [26, 27]. A change in the native state of cyt *c*, a shift of redox potential, and a better accessibility of the heme *c* crevice were detected upon binding to membranes, proteins, as well as synthetic negative surfaces [28-31]. Complexes between cyt *c* and a hydrophobic, negatively charged protein were described from which a part of cyt *c* was released into the reaction mixture in the reduced form [32]. The redox properties of cyt *c* bound in complexes changed on varying the cyt *c* to reactant protein ratio [33]. These effects may be reconciled with the results of our experiments obtained in the mixture of cyt *c* and WT preparations without an electron donor. For these experiments, conditions could be set favoring adduct conformation leading, on dynamic equilibrium, to an appearance of reduced cyt *c* in the reaction mixture. The process would seemingly accelerate the forward electron transfer from cyt c_1 to cyt *c*. The interpretation would be consistent with observations made with mitochondrial bc_1 [5, 9, 10]. In media of high ionic strength the value of K_{eq} = 3.3 for the electron transfer between the purified ferrocytochrome c_1 and ferricytochrome *c* was determined. At low ionic strength and in the presence of the acidic subunit (hinge) protein the K_{eq} values were about 40% higher than in its absence [5]. The idea suggested in this paper would be in accordance with findings of a

dynamic equilibrium between the bound and free cyt *c*. At low ionic strength and a pH near 7, the self-exchange reaction of cyt *c* is limited by electrostatic repulsive work terms. When bound to negative surfaces, the rate of electron exchange reaction increases dramatically, obviously due to increased frequency of productive collision among cyt *c* molecules [34]. This may account for the observed appearance of the reduced cyt *c* in the reaction mixture. It seems justified to conclude that coulombic effects have a regulatory role in the actual rate of electron transfer reactions between cyt c_1 and cyt *c*. In this way, a preference for electron transfer to the bound cyt *c* molecule may be established; this prevents dissipation of electrons among other redox carriers.

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