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Novel cyanide inhibition at cytochrome c_1 of *Rhodobacter capsulatus* cytochrome bc_1

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Abstract

Oxidized cytochrome c_1 in photosynthetic bacterium *Rhodobacter capsulatus* cytochrome bc_1 reversibly binds cyanide with surprisingly high, micromolar affinity. The binding dramatically lowers the redox midpoint potential of heme c_1 and inhibits steady-state turnover activity of the enzyme. As cytochrome c_1, an auxiliary redox center of the high-potential chain of cytochrome bc_1, does not interact directly with the catalytic quinone/quinol binding sites Q_o and Q_i, cyanide introduces a novel, Q-site independent locus of inhibition. This is the first report of a reversible inhibitor that manipulates the energetics and electron transfers of the high-potential redox chain of cytochrome bc_1, while maintaining quinone substrate catalytic sites in an intact form.

Keywords: Cytochrome bc_1; Cyanide inhibition; Heme; Electron transfer; *Rhodobacter capsulatus*

1. Introduction

Quinonoid inhibitors have provided the traditional means for teasing apart and isolating the string of connected electron transfers within the cytochrome bc_1. They have revealed two distinct quinone/quinol binding sites: stigmatellin and myxothiazol inhibited Q_o at which quinol is normally oxidized, and antimycin inhibited Q_i, at which quinone is normally reduced (Fig. 1) [1,2]. Furthermore, by displacing quinone at the binding sites in X-ray crystallographic structures [3–8], they approximate the position of the more loosely bound and so far unresolvable quinol in the Q_o site. However, unlike the reversible heme ligand inhibitors used with great effect by Babcock and others (for example see Ref. [9]), quinonoid inhibitors permanently displace quinone substrates and make it frustratingly impossible to observe and describe the natural engineering of the Q site catalytic events themselves. To date, no reversible inhibitors that bind outside the Q_o and Q_i sites have been found.

Here, we borrow a page from classical cytochrome oxidase inhibition studies and describe a novel way of manipulating redox cofactor properties in cytochrome bc_1. We show that oxidized cytochrome c_1 in the photosynthetic bacterium *Rhodobacter* (*Rba.*) *capsulatus* cytochrome bc_1 reversibly binds cyanide (cyt c_1-CN) with surprisingly high affinity and this binding shifts the heme redox midpoint potential dramatically. Such reversible inhibition allows the dissection of sequential electron transfers to be extended to the high-potential redox chain of cytochrome bc_1 without sabotaging Q_o site activity.

2. Materials and methods

Chromatophore membranes and cytochrome bc_1 were isolated and purified from *Rba. capsulatus* as described in Refs. [10,11]. All experiments were performed in 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, at room temperature. Optical spectra were recorded on a Perkin-Elmer UV/vis

Abbreviations: CN, cyanide; cyt c_1-CN, cytochrome c_1 with bound cyanide; Q_o and Q_i sites, quinol oxidation and quinone reduction sites of cytochrome bc_1; DBH_2, 2,3-dimethoxy-5-decyl-6-methyl-1,4-benzohydroquinone; 2Fe2S, two iron–two sulfur cluster; *Rba.*, *Rhodobacter*

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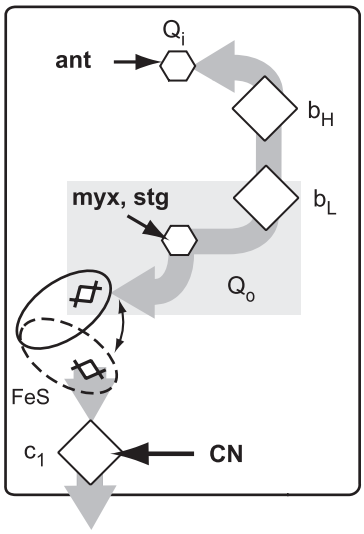

Fig. 1. Sites of inhibition in high- and low-potential redox chains of cytochrome bc_1. Conventional quinonoid inhibitors specifically replace quinones in the Q_o site (stigmatellin, stg; myxothiazol, myx) or in the Q_i site (antymycin, ant). Binding of cyanide (CN) to oxidized cytochrome c_1 introduces a new, Q_o and Q_i site-independent, site of inhibition.

spectrophotometer Lambda 20. To obtain difference spectra for *c*- and *b*-type cytochromes, samples were first oxidized by an addition of potassium ferricyanide (to a final concentration of 20 μM) and then reduced using sodium ascorbate (added to a final concentration of 0.2 mM) or a minimal amount of solid, fresh sodium dithionite. To obtain difference spectra of cyanide-treated cytochrome bc_1, oxidized samples were first treated with excess of KCN (see Fig. 3) and allowed to equilibrate with KCN for 5 h before ascorbate or dithionite was added.

To determine the binding constant for cyanide, the ferricyanide-oxidized samples containing 5 μM of purified cytochrome bc_1 were incubated with various amounts of KCN overnight to allow a relatively complete equilibration of the protein with the ligand [12]. After this incubation, the ascorbate *minus* ferricyanide optical redox difference spectrum for each KCN concentration was obtained and the fraction of ascorbate-reducible cytochrome c_1 was estimated from the amplitude of absorbance change 553–540 nm in the α-region. Similar titrations were performed with purified chromatophore membranes.

For observing the kinetics of cyanide release from cyt c_1-CN, a sample of 0.3 ml of oxidized cytochrome bc_1 (30 μM) was equilibrated with excess of KCN (1 mM) for 5 h. The sample was then passed through PD-10 desalting column (Pharmacia) equilibrated with 50 mM Tris–HCl, pH 8, 100 mM NaCl. Following the removal of excess of KCN, the sample was reduced with excess ascorbate (added to a final concentration of 2 mM) and the spectral changes in the α-region were recorded over time to monitor the recovery of the ascorbate-reducible component.

Steady-state enzymatic activity of cytochrome bc_1 was assayed by measuring 2,3-dimethoxy-5-decyl-6-methyl-1,4-benzohydroquinone (DBH_2)-dependent reduction of mitochondrial horse cytochrome *c* as described in Refs. [10,13], and the rate of cytochrome *c* reduction was related to the molar content of cytochrome c_1. Activities were assayed at room temperature in 50 mM Tris pH 8, 100 mM KCl, 0.1 g/l dodecyl maltoside with 8 nM cytochrome bc_1. For cyanide titration, samples containing 1.6 μM cytochrome bc_1 pre-incubated overnight without and with increasing concentrations of KCN were considered stock solutions for the enzymatic assay. In the assay, they were added to the reaction mixture containing the same concentration of KCN as that used in each stock solution. Undiluted stock solutions were also used to determine the amount of the cyt c_1-CN bound form in the ascorbate-reducibility test, as described above.

3. Results

Native cytochrome bc_1 has three heme redox components: high-potential heme c_1 and two low-potential hemes *b*, b_L and b_H. In *Rba. capsulatus* the redox potentials of these hemes at pH 7 are: +320 mV for heme c_1, +40 mV for heme b_H and −120 mV for heme b_L [13]. The large difference in the redox potentials between heme c_1 and hemes *b* allows us to perform simple tests for binding of the cyanide with the use of oxidants and reductants that distinguish between high- and low-potential redox components: ferricyanide oxidizes all three heme components, ascorbate reduces only heme c_1, dithionite reduces both hemes *b* in addition to heme c_1.

Fig. 2 shows the effect of adding cyanide to fully oxidized cytochrome bc_1. The clear red shifts both in the Soret and α-region with transitions at 403, 422, 521 and 548 nm are typical of cyanide binding to heme, while the α-band changes around 550 nm indicate that cyanide binds to oxidized heme c_1 rather than either of the hemes *b*. Indeed, the observation that ascorbate-reduced cytochrome bc_1 does not show spectral changes after addition of cyanide (not shown) confirms that oxidized hemes *b* do not bind cyanide.

The binding of cyanide to ferricytochrome c_1 is expected to have a large effect on the redox midpoint potential of heme c_1. The optical redox difference spectra of isolated cytochrome bc_1 shown in Fig. 3a,b reveal that the cyt c_1-CN indeed has a severely lowered redox potential. The spectrum of the native form of the complex is characterized by the presence of both ascorbate- and dithionite-reducible components (Fig. 3a, dashed and solid line, respectively). The binding of cyanide eliminates the ascorbate-reducible component (Fig. 3b, dashed line) and causes dithionite to reduce both hemes *b* prior to heme c_1 (Fig. 3b, solid line). The shoulder at 552–554 nm in the dithionite reduced spectrum, clearly seen in the native form

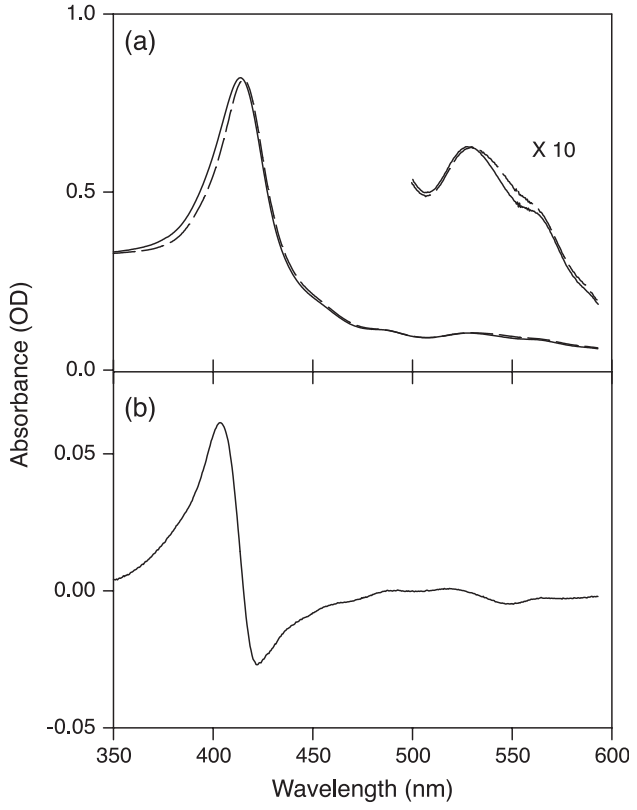

Fig. 2. Cyanide-induced optical spectral changes in purified cytochrome bc_1. (a) Absorption spectra of ferricyanide oxidized bc_1 before (solid line) and after addition of 2 mM KCN (dashed line), (b) ferricyanide oxidized *minus* ferricyanide oxidized-cyanide bound difference spectrum.

(Fig. 3a, solid line) appears in the sample treated with cyanide only after initial reduction of hemes *b* and prolonged incubation with dithionite (not shown). This indicates that cyanide binds to the heme c_1 reversibly and lowers its midpoint potential dramatically, making it much more difficult to reduce. Fig. 3c and d demonstrates that cyanide has the same effect on the redox properties of cytochrome bc_1 in chromatophore membranes. Because no *c*-type absorption spectrum appears in cyanide and ascorbate treated chromatophores (Fig. 3d, dashed line), cyanide is likely to interfere with the ascorbate reducibility of all the high potential *c*-type cytochromes in chromatophores, including cytochrome c_2 and c_y. This would agree with the earlier observations that some bacterial cytochromes *c* display a much more increased propensity to bind exogenous strong field ligands than their mitochondrial counterparts [14,15] (see discussion below).

The cyanide-induced shift in the redox potential of cytochrome c_1 was used to estimate the binding constant (K_d) for cyanide. This approach combined the titration of cytochrome c_1 with cyanide with the ascorbate reducibility test, and was based on the assumption that ascorbate reduces only that portion of cytochrome c_1 that did not bind cyanide when ferricytochrome c_1 was exposed to react with it. Thus, in the effective range of cyanide concentrations, the amount of ascorbate-reducible cytochrome c_1 will depend on the concentration of cyanide used. As shown in Fig. 4, the fraction of ascorbate-reducible cytochrome c_1 (i.e. fraction of the "free" form) decreases with increasing amount of cyanide and the titration profile allows us to estimate a K_d of approximately 25 μM for isolated cytochrome bc_1, and a similar value for chromatophores. It should be noted that our initial experiments showed that the binding of cyanide is very slow and occurs within hours (data not shown). Therefore, an extended overnight incubation with cyanide was allowed before the ascorbate reduction to assure more or less complete equilibration for all but the smallest cyanide concentrations.

The ascorbate-reducibility test was also used to monitor the kinetics of release of cyanide from cyt c_1-CN. In this assay, the recovery of the cytochrome c_1 ascorbate reducibility occurs parallel to the release of cyanide from the heme-binding pocket. The time-course of this process is shown in Fig. 5. Given that the kinetics of cyanide binding/release is much slower that the reduction of cytochrome c_1 by ascorbate, we estimate the k_{off} of 0.0019 min^{-1} for the release of cyanide. This k_{off} value and the K_d of 25×10^{-6} M, gives the second-order rate of binding of cyanide to cytochrome c_1, k_{on}, of 1.2 M^{-1} s^{-1}.

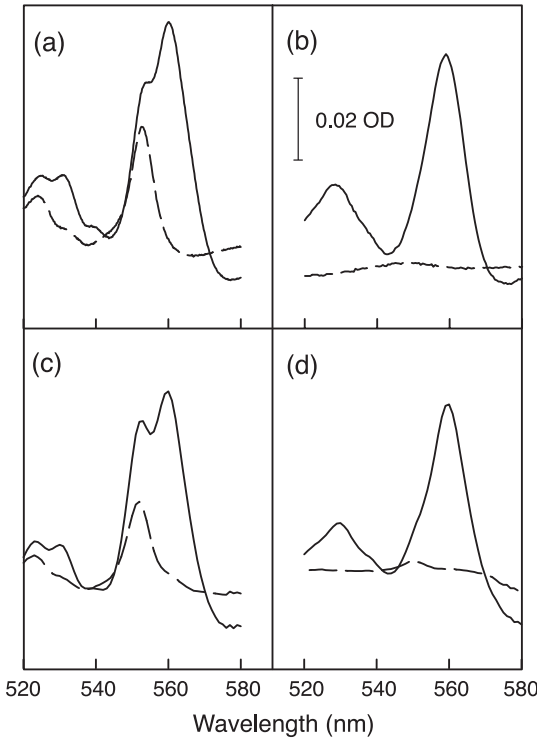

Fig. 3. Effect of cyanide on the redox properties of cytochrome c_1 in isolated cytochrome bc_1 (a, b), and cytochromes *c* in chromatophores (c, d). Ferricyanide oxidized samples were either untreated (a, c) or treated with 1 mM KCN (b, d) and then reduced by ascorbate or dithionite. Solid and dashed lines correspond to dithionite *minus* ferricyanide and ascorbate *minus* ferricyanide optical redox difference spectra, respectively.

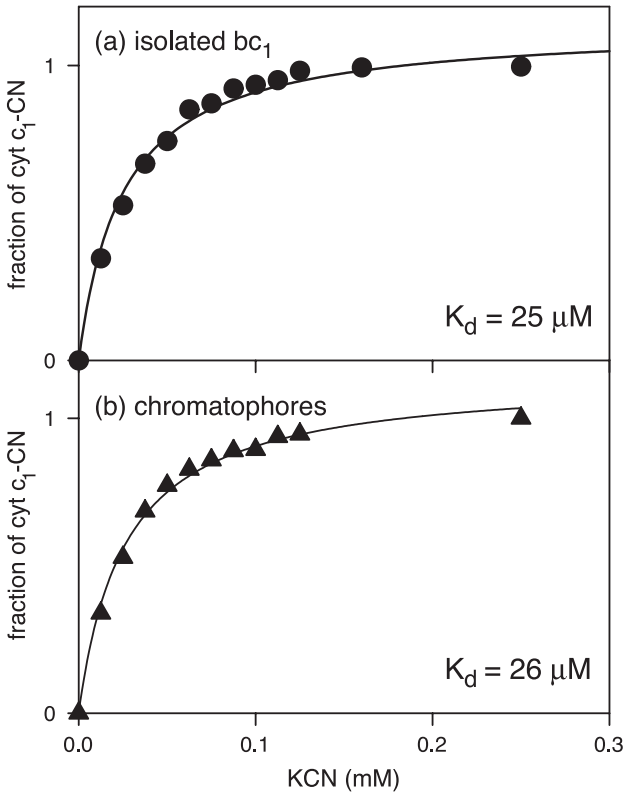

Fig. 4. Determination of the binding constant for cyanide in oxidized cytochrome c_1 in isolated bc_1 complexes (a) and chromatophores (b) using the ascorbate reduction assay described in materials and methods. Fractions of ascorbate non-reducible cytochrome c_1 with bound cyanide (cyt c_1-CN) are plotted versus the concentration of cyanide.

Fig. 6 shows the inhibition of ubiquinol-cytochrome *c* reductase activity by cyanide in an enzymatic steady-state turnover assay using catalytic amounts of purified cytochrome bc_1. The activity of the enzyme decreases linearly with increasing concentration of cyt c_1-CN form with a slight deviation from the linearity at low concentrations of cyanide (below 10 μM). However, cyanide does not completely inhibit the enzymatic activity of cytochrome bc_1; even at high concentrations of cyanide (where the concentration of cyt c_1-CN is maximal), a significant (22%) activity of the complex is observed. For comparison, only 2% activity is observed when cytochrome bc_1 is fully inhibited with antimycin.

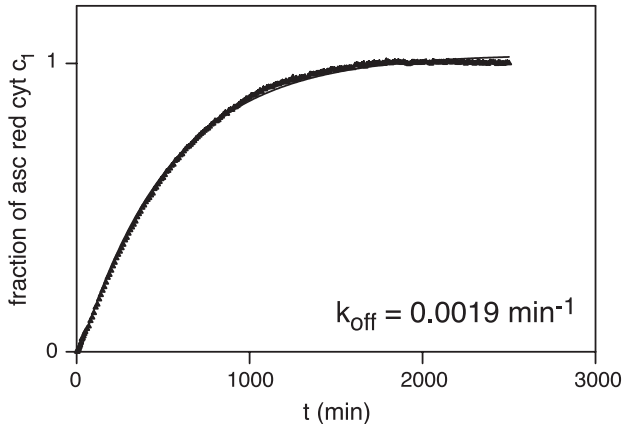

Fig. 5. Kinetics of cyanide release from cytochrome c_1 in isolated bc_1 complexes monitored as a recovery of ascorbate-reducible component (asc red cyt c_1) (553–540 nm) in the spectrum.

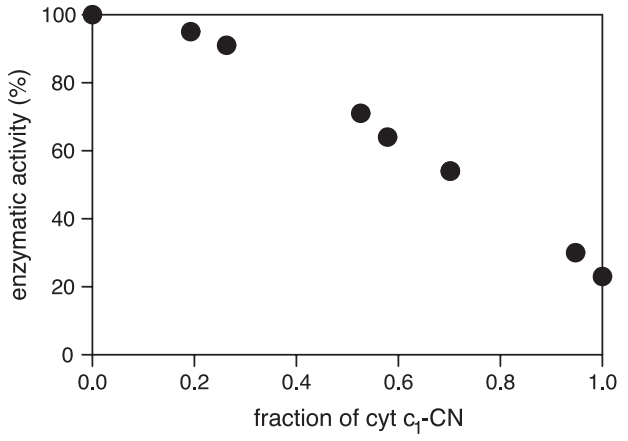

Fig. 6. Effect of cyanide on the enzymatic ubiquinol-cytochrome *c* reductase activity of cytochrome bc_1. Activities are expressed as a percentage of the activity without inhibitor (measured turnover rate 210 s^{-1}) and plotted versus the fraction of cyt c_1-CN (bound form) in the samples used in the assay.

4. Discussion

Bacterial cytochrome bc_1 displays a high degree of homology with mitochondrial complex III. In both systems, three redox cofactor containing subunits, cytochrome c_1, 2Fe–2S subunit and cytochrome *b*, form similarly assembled and operating catalytic core. Within this core, the two quinol/quinone binding sites, by providing an environment for catalytic chemical reactions and defining critically important, but different levels of stability for the semiquinone state, are expected to display the highest degree of structural conservation among various species. On the other hand, the parts more remote from the quinone binding sites may have larger degree of conformational freedom allowing some structural variation among species to occur. Indeed, the most significant differences can be found within the most peripheral subunit, cytochrome c_1, which interacts directly only with the physiological partners of cytochrome bc_1 (like cytochromes *c*) and the mobile 2Fe–2S domain (Fig. 1). For example, some bacterial cytochromes c_1 have additional structure–function related insertions in the loop regions, while the homologous cytochrome b_6f in chloroplasts, replaces the entire cytochrome c_1 with structurally different cytochrome *f*. Recently, we have recognized that cytochrome c_1 of the photosynthetic bacterium *Rba. capsulatus* has an unusual structural characteristic relying on a disulfide bond to maintain the proper heme axial

ligation and secure the functional heme redox potential [16]. Such structural variability in cytochrome c_1 may be at the root of differences in cyanide sensitivity; bovine mitochondrial cytochrome bc_1 does not bind cyanide even at very high levels (Rich, P.R., University College London, personal communication).

The behavior of *Rba. capsulatus* cytochrome c_1 is consistent with a model in which an added ligand binds to the heme iron by displacing one of its native coordinating ligands [14,15,17–19]. In cytochrome *c*, the transition from reduced to oxidized form is known to involve a dynamic rearrangement which weakens the strength of methionine sixth axial ligation and permits its replacement by exogenous strong field ligands. This site is therefore the most plausible region of interaction of cytochrome c_1 with cyanide. Such perturbation of heme axial ligation correlates with a decrease of hundreds of mV in the redox potential of cyt c_1-CN (Fig. 3) in analogy with cyanide-induced midpoint drops in other heme proteins [20,21]. The perturbation, however, does not cause an irreversible damage to the protein; the release of cyanide in experimental conditions of Fig. 5 restores the native spectral and redox properties of cytochrome c_1 as indicated by the recovery of the native-like ascorbate-reducible component in the spectrum of cytochrome bc_1 (not shown).

The propensity to bind exogenous strong field ligands observed in certain other cytochromes *c* (in particular bacterial cytochromes c_2) has been related to the dynamic process of the opening of the heme crevice to vacate the sixth coordination for interaction with exogenous ligands [14,15]. The rapid interconversion between the closed and opened forms is probably part of the natural protein dynamics and originates largely from the increased conformational mobility of the sixth axial ligand domain. In *Rba. capsulatus* cytochrome c_1, this region encompasses the disulfide anchored extra loop, which appears to control the integrity of the heme-binding pocket and secure the proper heme axial ligation [16]. Such a strategy for governing heme-ligand reactivity and conformational stability has not been seen in other *c*-type cytochromes and may be one of the factors responsible for the unusual ligand binding properties of this cytochrome. Moreover, the cyanide binding and release of cytochrome c_1 are extremely slow indicating that, despite its apparent accessibility for the external ligand, the heme coordination site remains severely hindered. The disulfide-anchored loop may provide a steric barrier that protects the interior of the heme-binding pocket, yet has some degree of mobility allowing limited access from outside.

Despite a cyanide-induced cytochrome c_1 redox midpoint potential drop below ascorbate reducibility, which makes 2Fe2S to cyt c_1-CN electron transfer energetically unfavorable, steady-state ubiquinol/cytochrome *c* oxidoreductase activity is still observable at approximately 20% of native activity. This activity occurs even when essentially all cytochrome c_1 exhibits the spectral signature of cyt c_1-CN. Clearly, under steady-state conditions Q_o site reduced 2Fe2S is capable of defeating the cyanide inhibition.

It is conceivable that cyanide bound to heme c_1 might briefly and incompletely dissociate from oxidized heme c_1 on the time scale of catalytic turnover, analogous to classic photo-dissociated heme ligands that remain near the heme pocket and re-associate relatively quickly (for example, see Ref. [22]). This might enable normal 2Fe2S to heme c_1 electron transfer, albeit at reduced rates. However, under these circumstances, dissociated cyanide would have to stand ready to rebind quickly to cytochrome c_1, when reoxidized by cytochrome *c*, to continue inhibition of turnover. This rebinding would have to be much faster than the binding rates we measure from free solution.

However, we favor an alternative in which energetically unfavorable electron transfer from 2Fe2S to cyt c_1-CN may occur without cyanide dissociation. Analogous electrochemical reduction of CN-bound heme has been probed by cyclic voltammetry with myoglobin [20]. Redox midpoint potentials for the CN-bound and CN-free myoglobin heme were resolved, and redox associated cyanide dissociation and association rates were measured. In cytochrome bc_1, the rate of the uphill electron transfer from 2Fe2S to cyt c_1-CN will depend on the redox potential of cyt c_1-CN and the inherent rate of the downhill electron transfer from cyt c_1-CN to 2Fe2S. The rate of native electron transfer between 2Fe2S and cytochrome c_1 has been reported to range from roughly 60,000 to 6000 s^{-1} [23], with the latter rate consistent with the 16 Å tunneling distance at closest approach in bc_1 crystals, assuming typical reorganization energies [24]. An energetic penalty for the 2Fe2S to cyt c_1-CN reaction of 2.2–3.2 orders of magnitude (130–200 mV) is required to slow the rate to our inhibited steady-state value of about 40 s^{-1}. The corresponding cyt c_1-CN redox midpoint potentials in the range of 80–150 mV are just what is required to make ascorbate reduction unfavorable.

The binding of cyanide to the native ferricytochrome c_1 of *Rba. capsulatus* cytochrome bc_1 is a curiously late finding, and provides a caution to those who view cyanide as a simple selective means for cytochrome oxidase inhibition, especially at high cyanide concentrations. Cyanide provides the first cytochrome bc_1 inhibitor outside the Q_o and Q_i ubiquinone sites, and its reversibility promises to be a useful tool to manipulate the extended high-potential electron transfer chains in cytochrome bc_1 to investigate the dynamics of Q_o site catalysis and 2Fe2S motion in this key component of photosynthetic and respiratory chains.

Acknowledgements

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