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Peculiarities of Cyanide Binding to the ba_3-Type Cytochrome Oxidase from the Thermophilic Bacterium *Thermus thermophilus*

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Abstract—Cytochrome *c* oxidase of the ba_3-type from *Thermus thermophilus* does not interact with cyanide in the oxidized state and acquires the ability to bind heme iron ligands only upon reduction. Cyanide complexes of the reduced heme a_3 in cytochrome ba_3 and in mitochondrial aa_3-type cytochrome oxidase are similar spectroscopically, but the a_3^{2+}–CN complex of cytochrome ba_3 is strikingly tight. Experiments have shown that the K_d value of the cytochrome ba_3 complex with cyanide in the presence of reductants of the enzyme binuclear center does not exceed 10^{-8} M, which is four to five orders of magnitude less than the K_d of the cyanide complex of the reduced heme a_3 of mitochondrial cytochrome oxidase. The tightness of the cytochrome ba_3 complex with cyanide is mainly associated with an extremely slow rate of the ligand dissociation ($k_{off} \leq 10^{-7}$ sec^{-1}), while the rate of binding ($k_{on} \sim 10^2$ $M^{-1} \cdot sec^{-1}$) is similar to the rate observed for the mitochondrial cytochrome oxidase. It is proposed that cyanide dissociation from the cytochrome ba_3 binuclear center might be hindered sterically by the presence of the second ligand molecule in the coordination sphere of Cu_B^{2+}. The rate of cyanide binding with the reduced heme a_3 does not depend on pH in the neutral area, but it approaches linear dependence on H^+ activity in the alkaline region. Cyanide binding appears to be controlled by protonation of an enzyme group with $pK_a = 8.75$.

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Key words: cytochrome oxidase ba_3, cyanide, hemoproteins, oxygen reducing center, *Thermus thermophilus*

The majority of living organisms get energy via respiration, i.e. reduction of molecular oxygen to water while oxidizing various physiological substrates. This multistage process is realized by the respiratory chain located in the inner mitochondrial membrane in eucaryotes and in the cytoplasmic membrane in bacteria. The final reaction of the respiratory chain is catalyzed by terminal oxidases, which most frequently are members of the so-called heme–copper family [1]. In enzymes of this type, the oxygen reduction center contains a high-spin heme and a closely located copper ion Cu_B that are cooperatively involved in the transfer of four electrons to an O_2 molecule with production of two molecules of water. The catalytic subunit I carrying the binuclear oxygen reducing center also contains a low-spin heme through which electrons enter the oxygen reduction center.

The second catalytic subunit is responsible for oxidizing the respiration substrate, which is usually represented either by a small water-soluble cytochrome *c* (in cytochrome *c* oxidases) or by a reduced form of hydrophobic quinone dissolved in the membrane (in quinol oxidases). In cytochrome oxidases, electrons from cytochrome *c* initially come onto the two-atom copper cluster Cu_A located on the membrane outer side. The subunit II of quinol oxidases does not contain copper, and the binding site for cytochrome *c* is substituted in it by the site for tight binding of quinol [2, 3].

In all studied oxidases of the heme–copper family, the exergonic transfer of electrons to oxygen is coupled with the endergonic transfer of protons across the coupling membrane, which results in generation of transmembrane electrochemical potential difference of H^+ ions, $\Delta\mu H^+$; then this form of energy can be used for different functions of the cell (synthesis of ATP, transfer of substances across the membrane, etc.). The transmembrane transfer of protons through the protein molecule is realized by "proton channels", which were predicted to exist in the enzyme more than 30 years ago [4-6] and confirmed upon resolution of the three-dimensional structure of several oxidases [7-9].

Abbreviations: DAD, diaminodurene; DM, dodecyl maltoside.
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Genes of great number of terminal oxidases (first of all, of bacterial ones) have been sequenced. A systematic analysis of amino acid sequences of subunit I [10-12] mainly based on comparison of the regions forming the proton channels resulted in subdivision of the heme–copper family into A, B, and C classes. The class A includes the best-studied "canonical" cytochrome *c* oxidases of the aa_3-type from mitochondria, *Paracoccus denitrificans*, and *Rhodobacter sphaeroides*, as well as the *bo′*-type quinol oxidase (bo_3) from *Escherichia coli*. Three-dimensional structures have been obtained by X-ray crystallography for all these enzymes [7, 8, 13] (reviewed [3]). An enzyme of the B class, the cytochrome oxidase ba_3 from *Thermus thermophilus*, has also been analyzed by X-ray crystallography [9]. The three-dimensional structure is not yet established for oxidases of the C class (bacterial cytochrome *c* oxidases of the cbb_3-type).

Comparison of the ba_3 cytochrome oxidase structure with those of the A-class oxidases reveals some significant differences in the binuclear center and proton channels. In all of these proteins, one can see three pores oriented across the membrane. In the A-class oxidases, the pores are denoted as K-, D-, and H-channels. Only the K-channel is partially homologous to its spatial analog in the ba_3 cytochrome. The pore positioned approximately like the D-channel has no characteristic conservative residues in the ba_3-type oxidase. The third channel-like structure of the cytochrome ba_3 (the Q-channel) has neither spatial nor amino acid similarity with the H-channel of canonical oxidases.

Distances between the high-spin heme a_3 and Cu_B and between the high-spin heme iron and its axial ligand His384 in the binuclear center of the cytochrome oxidase ba_3 are different from those in the A-class members; moreover, only one oxygen atom instead of two atoms is present between the heme a_3 and Cu_B in the fully oxidized cytochrome ba_3 [9, 14, 15]. The half-reduction potentials of the redox-groups also differ for the cytochrome oxidase ba_3 and the A-class oxidases [16, 17]. The oxidase ba_3 is characterized by the much more developed "oxygen channel", which is likely to serve not only for delivery of O_2 molecules into the binuclear center but also for releasing of produced H_2O molecules. In relation with the latter function, note that on the boundary between subunits I and II in cytochrome oxidase ba_3 structure there is no tightly bound magnesium ion, which is believed to play an important role in providing ways for releasing water and protons from the oxygen reducing center of canonical oxidases.

A question arises if differences in amino acid sequences and three-dimensional structures correspond to the differences in functional features and biochemical properties of heme–copper oxidases from different classes. In the present work interaction has been investigated of the oxidase ba_3 from *T. thermophilus* with cyanide, which is a classical inhibitor of the cytochrome oxidase reaction and binds to the heme a_3 high-spin iron ion. It is interesting that the fully oxidized cytochrome oxidase ba_3 is unable to bind exogenous ligands [18]. In the case of cyanide, the reaction occurs only in the presence of reductants of the binuclear center, and the optical absorption spectrum suggests that the produced complex corresponds to the a_3^{2+}–CN form, whereas for the A-class oxidases, production of a tight cyanide complex with the oxidized heme is typical (a_3^{3+}–CN). The oxidase ba_3 cyanide complex is shown to be extremely tight and its production is controlled by protonation of a group of the protein.

MATERIALS AND METHODS

Reagents and preparations. Chemical purity and special chemical purity reagents of domestic production were used; dodecyl maltoside (DM) from Sigma (USA), sperm whale myoglobin from Serva (Germany), and diaminodurene (DAD) (2,3,5,6-tetramethyl-*p*-phenylenediamine) from Fluka (USA) were also used.

Cytochrome oxidase was isolated from bovine heart mitochondria by Fowler's method [19]. The purified preparation of cytochrome oxidase ba_3 was isolated from *T. thermophilus* in the laboratory of T. Soulimane as described earlier [20].

The reduced state of DAD in the initial solution was maintained by addition of 1 mM ascorbate to 0.5 M DAD solution in alcohol. Cyanide was added from a freshly prepared solution of KCN (0.01-1 M), which was incubated on ice during the experiment. Sodium dithionite was added from 1 M solution prepared immediately before the addition.

Media. The majority of experiments with the cytochrome oxidase ba_3 were performed in medium containing 0.1 M Hepes/KOH (pH 7.6) and 0.05% DM. The pH dependence of the reaction with cyanide was studied in media containing 50 mM potassium phosphate, 0.05% DM (pH 6.0-8.6); Ches/KOH, 0.05% DM (pH 9.0-9.5).

Experiments with mitochondrial cytochrome oxidase were performed in medium containing 0.1 M Hepes/NaOH (pH 7.6), 0.05% DM, and 0.2 mM EDTA.

Spectroscopy. Optical absorption spectra were recorded using a Cary-300 instrument (Varian, USA) in a standard cuvette with 1 cm optical path. Absolute spectra were recorded with 2-nm slit width at scanning rate 2 nm/sec. Difference spectra were obtained by subtraction of successively recorded absolute spectra. Kinetic measurements were performed at 22°C using an SLM-Aminco 2000 spectrophotometer (USA) in dual wavelength mode with 2-nm slit width. Anaerobic conditions were created by bubbling argon through the sample solution for 20 min, and then the cuvette was tightly closed.

The majority of experiments were performed in the presence of 1-2 μM cytochrome oxidase in the cuvette.

Concentration of cytochromes was determined using the following values of extinction coefficients: $\varepsilon_{605\text{-}630\ nm}$ = 27 $mM^{-1}\cdot cm^{-1}$ for oxidase aa_3 (on the difference spectrum resulting upon reduction of the initially oxidized preparation with dithionite) [21]; $\varepsilon_{560\text{-}590\ nm}$ = 26 $mM^{-1}\cdot cm^{-1}$ for oxidase ba_3 (on the absolute spectrum of the fully reduced sample) [22]; $\varepsilon_{509\ nm}$ = 157 $mM^{-1}\cdot cm^{-1}$ for metmyoglobin (on the absolute spectrum of the oxidized sample) [23].

RESULTS

Cyanide-induced spectral changes in mitochondrial cytochrome oxidase of aa_3-type and in bacterial oxidase of ba_3-type. Figure 1 presents changes in absorption spectra of the mitochondrial oxidase aa_3 (a) and bacterial oxidase ba_3 (b) caused by addition of cyanide to the fully oxidized (dashed line) and to fully reduced (solid line) enzyme.

In the aa_3-type oxidized cytochrome oxidase (Fig. 1a), cyanide induced appearance of an intense difference spectrum ($\Delta A_{432\text{-}411}$ ~ 45 $mM^{-1}\cdot cm^{-1}$) typical for the shift of the Soret band to longer wavelengths and a relatively small increase in the extinction in the visible part of the spectrum. These changes are specific for the transition of oxidized high-spin heme a_3^{3+} to the low-spin state. However, addition of cyanide to the ba_3-type oxidized cytochrome oxidase (Fig. 1b) caused only small asymmetric changes in the Soret region with the maximum at 441 nm and increased the α-band at 592 nm. These changes reflect the interaction of cyanide with a small portion of the cytochrome ba_3 in which there occurs heme a_3 transition to the reduced state (compare the spectra in Fig. 1b), whereas no binding of the ligand with the oxidized heme a_3 is observed.

In contrast, interaction of cyanide with the reduced forms of both oxidases causes similar spectral changes (Figs. 1a and 1b, solid lines). Responses in the Soret region (long wavelength shift) and in the visible region (short wavelength shift) were comparable in size. The responses in the visible region are shown in more detail in Fig. 1c. In the aa_3 cytochrome oxidase (dashed line), the reduced heme a_3 binding with cyanide was accompanied by appearance of an asymmetric difference spectrum ($\Delta\varepsilon_{589\text{-}650}$ = 15 $mM^{-1}\cdot cm^{-1}$, $\Delta\varepsilon_{589\text{-}610}$ = 23 $mM^{-1}\cdot cm^{-1}$, half-width = 9.4 nm). In the difference spectrum of the ba_3-type enzyme (solid line) the response in the α-region was slightly higher in amplitude, narrower by ~2 nm, and displaced by 3-4 nm to longer wavelengths ($\Delta\varepsilon_{592\text{-}670}$ = 20.3 $mM^{-1}\cdot cm^{-1}$, $\Delta\varepsilon_{592\text{-}614}$ = 27.6 $mM^{-1}\cdot cm^{-1}$, half-width = 7.6 nm).

Difference in cyanide interaction with the reduced oxidases aa_3 and ba_3. Despite the spectral similarity, cyanide complexes of the reduced heme a_3 in the cytochrome oxidases aa_3 and ba_3 are significantly different in both the tightness and rate of formation (Fig. 2). In

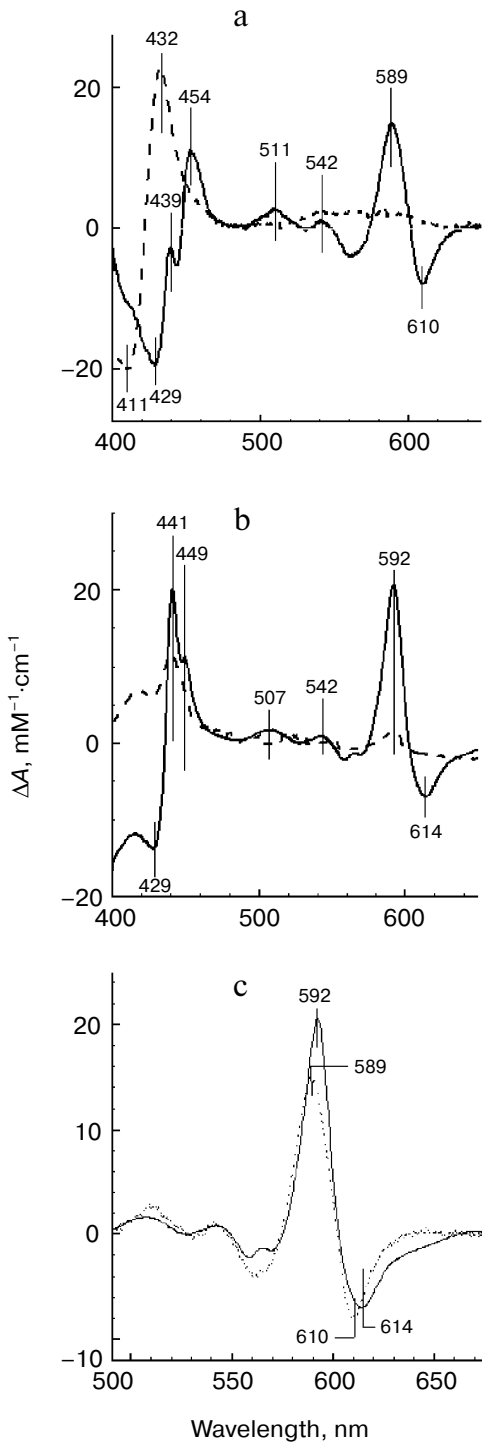

Fig. 1. Cyanide-induced spectral changes in cytochrome oxidases aa_3 and ba_3. Difference spectra of oxidized and dithionite-reduced oxidases recorded after incubation for 20 min in the presence of 10 mM KCN. Along the ordinate axis optical absorption normalized to the enzyme concentration is shown. a) Oxidized (dashed line) and reduced (solid line) cytochrome oxidase aa_3 from bovine heart mitochondria. b) Oxidized (dashed line) and reduced (solid line) cytochrome oxidase ba_3 from *T. thermophilus*. c) Comparison of the cyanide-induced spectral changes in the reduced cytochrome oxidases ba_3 (solid line) and aa_3 (dashed line) in the visible region.

the presence of 100 μM KCN, the reduced ba_3-type enzyme completely binds with cyanide (Fig. 2a, spectrum *1*), whereas the reduced aa_3-type enzyme during this time forms only one third of the complex (spectrum *2*).

Typical kinetics of cyanide binding with the two cytochrome oxidases are presented in Fig. 2b. Addition of 50 μM cyanide to the reduced oxidase ba_3 results in a relatively slow formation of the complex (τ is about 3 min), but the reaction goes to completion (curve *1*). In a similar experiment with the reduced oxidase aa_3 equilibrium was established during the time of mixing but the yield of cyanide complex was only ~10% (curve *2*).

Figure 3 shows that the response to cyanide addition depends on the ligand concentration. In the presence of 2-50 μM KCN, reduced oxidase of ba_3-type (Fig. 3, a and b) completely converts to the cyanide complex. The reaction has mono-exponential kinetics (Fig. 3a) and is characterized by the rate constant of ~110 $M^{-1}\cdot sec^{-1}$ (Fig. 3b). In experiments with the reduced oxidase aa_3 (Fig. 3, c and d), an increase in the cyanide concentration in the range of 70-500 μM causes an increase in the final response (Fig. 3c) and the complex is fully formed only at millimolar concentrations of the ligand. The concentration dependence of the product formation (Fig. 3d) shows that cyanide complex of the reduced cytochrome oxidase aa_3 has a stoichiometry of 1 : 1 (see inset) and is characterized by K_d of ~0.35 mM, which is in good agreement with the literature [24-26].

On manual mixing it was impossible to resolve the kinetics of the interaction of reduced aa_3 oxidase with cyanide at concentrations >500 μM ([KCN] > K_d) (Fig. 3c). Note that when the reaction is performed at cyanide concentrations significantly lower than K_d, the observed k_{obs} of the adduct formation is dominated by the complex dissociation rate constant k_{off}. According to our data, k_{off} is approximately 0.045 sec^{-1} (the intercept of the plot on the ordinate, see inset), which is close to the value of 0.05 sec^{-1} reported in the work [24].

Evaluation of the affinity of cyanide binding with reduced ba_3 cytochrome oxidase. Figure 3a shows that within 15-20 min after the addition of 50 μM cyanide, the reduced oxidase ba_3 binds the ligand completely, which suggests a very high affinity of the reduced heme a_3 for the ligand. This is confirmed by formation of the oxidase complex with cyanide in the presence of ferrocyanide without addition of KCN. Ferrocyanide is a weak donor of electrons (half-reduction potential E_0' of the ferrocyanide/ferricyanide pair is about +420 mV [27]) and can also serve a donor of the cyanide group. Dissociation of $[Fe(CN)_6]^{4-}$ by all six steps is characterized by K_d ~ 10^{-24} M [28], however, the first step of complex dissociation may result in equilibrium concentration of the ligand of about 10^{-5} M (there are examples of mitochondrial cytochrome oxidase inhibition in the presence of ferrocyanide due to appearance of free cyanide in the medium [29]). Figure 4a shows changes in the absorption spectrum of oxidase ba_3 during incubation in the presence of 5 mM ferrocyanide. Both hemes of the cytochrome are reduced about 30% within the first minutes, and then during several tens of minutes the cyanide complex of the heme a_3^{2+} is formed and heme *b* goes oxidized. Upon incubation for 1-2 h, the cyanide complex is formed vir-

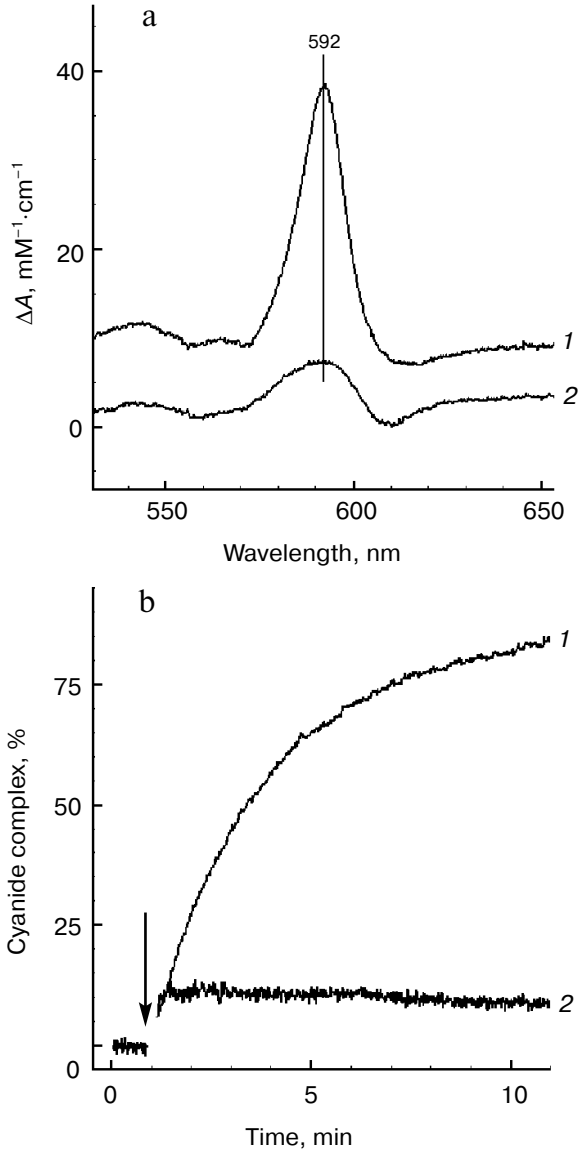

Fig. 2. Difference in the affinities of reduced cytochrome oxidases aa_3 and ba_3 for cyanide. a) Difference absorption spectra obtained upon incubation for 1 h of the reduced oxidases ba_3 and aa_3 (spectra *1* and *2*, respectively) in the presence of 100 μM KCN. Reduced DAD (2 mM, aerobic conditions, spectrum *1*) or dithionite (anaerobiosis, spectrum *2*) are used as electron donors. b) Kinetics of formation of cyanide complex of the reduced oxidases ba_3 and aa_3 (curves *1* and *2*). The arrow shows the addition of 50 μM KCN. Along the ordinate axis the enzyme fraction is shown (determined by the difference in absorption at 592-575 nm for oxidase ba_3 and at 589-610 nm for oxidase aa_3) which forms the complex with cyanide. Other conditions are similar to those in Fig. 2a.

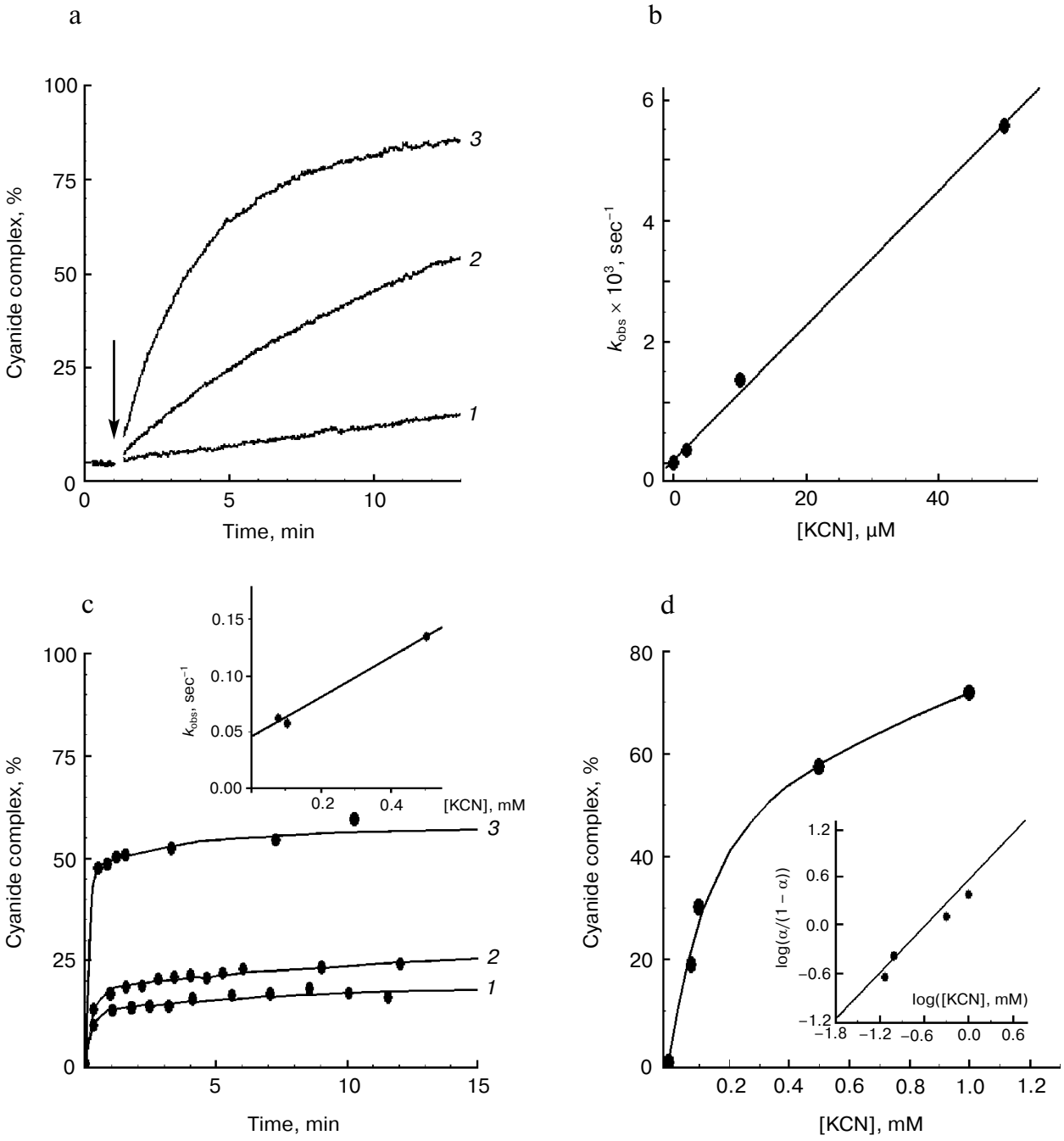

Fig. 3. Evaluation of tightness of cyanide complexes of reduced cytochrome oxidases aa_3 and ba_3. a) Kinetics of formation of ba_3 oxidase cyanide complex. The arrows show the addition of KCN to 2 (*1*), 10 (*2*), and 50 μM (*3*). Other conditions are the same as in Fig. 2b. b) Dependence of the rate of ba_3 oxidase cyanide complex formation on ligand concentration. Along the ordinate axis, values are shown of the apparent rate constant k_{obs} determined on approximation of the kinetics presented in Fig. 3a by the function $y = y_0 + A \cdot (1 - e^{-x \cdot k_{obs}})$ using the Origin 7.0 program. c) Kinetics of aa_3 oxidase cyanide complex formation. Experimental values (optical absorption difference at 589-610 nm) are obtained from successively recorded absolute spectra of the preparation incubated in the presence of 74 (*1*), 100 (*2*), and 500 μM KCN (*3*). Other conditions are the same as in Fig. 2b. In the inset, dependence of the apparent constant of the reaction rate on the cyanide concentration is shown (determined as in Fig. 3b). d) Dependence of aa_3 oxidase cyanide adduct yield on the ligand concentration. The fraction of the enzyme cyanide complex under equilibrium conditions at the given cyanide concentration was determined from the experimental data presented in Fig. 3c. In the inset these results are presented on the logarithmic scale (Hill plot; α is the cyanide complex fraction). The linear function drawn through the experimental points is taken from the work [24].

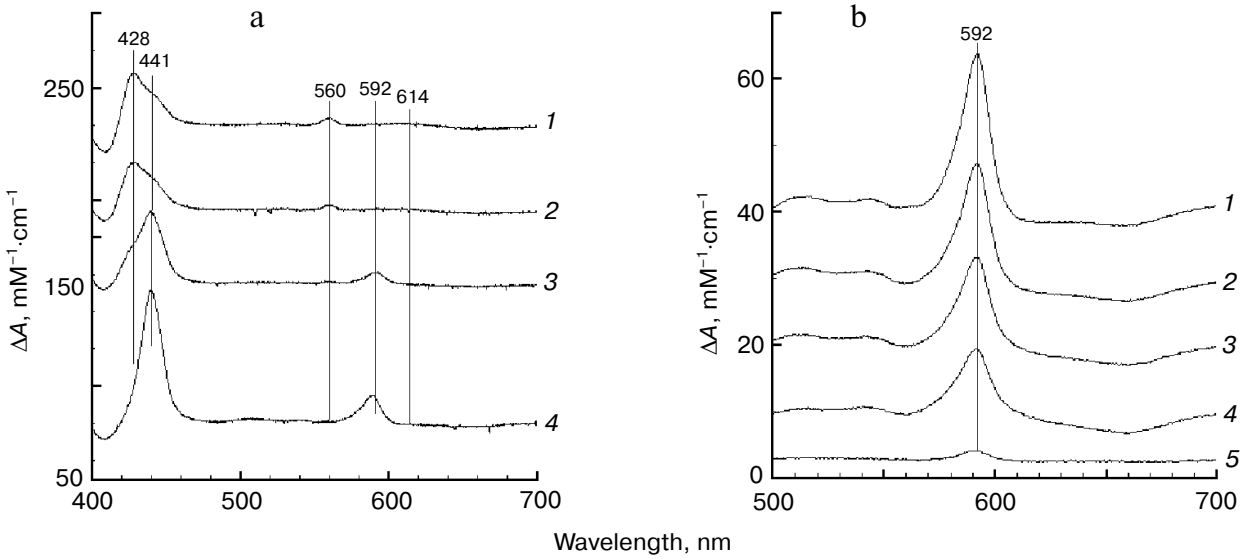

Fig. 4. Formation of the cytochrome ba_3 cyanide complex in the presence of ferrocyanide. a) Difference spectra of optical absorption obtained on incubation of the initially oxidized oxidase ba_3 (3 μM) in the presence of 5 mM $K_4Fe(CN)_6$. Spectra *1-4* were recorded 1, 10, 30, and 60 min after the addition of ferrocyanide. b) Redox titration of the oxidase ba_3 cyanide complex with the ferrocyanide/ferricyanide couple. The visible difference spectra (vs. the oxidized preparation) of the sample prepared in the previous experiment are presented. Spectrum *1* was recorded after 120-min incubation of oxidase ba_3 with ferrocyanide after the complete formation of the cyanide complex. Spectra *2-5* were recorded upon completion of the spectral changes caused by addition to the system of increasing concentrations of $K_3Fe(CN)_6$ (to the ratios of ferrocyanide/ferricyanide equal to 3 : 1, 1 : 1, 1 : 2, and 1 : 7, respectively).

tually completely, and this suggests that the cytochrome ba_3 heme a_3^{2+} and Fe^{2+} have comparable affinities for cyanide. Oxidation of heme *b* in the cyanide-bound enzyme can be explained by a decrease in its redox potential upon heme a_3 reduction as a result of the anti-cooperative interaction between the hemes typical of the oxidases of the heme–copper family.

The effective value of the heme a_3 E_0' in the cyanide complex can be assessed by incubation of the initially oxidized cytochrome ba_3 in the presence of the ferrocyanide/ferricyanide redox pair (Fig. 4b). The presented difference spectra (vs. the oxidized sample) were recorded after the establishment of equilibrium in the system. The reduced heme a_3 seems to be half-bound with cyanide at the ferrocyanide/ferricyanide ratio within a range of 1 : 1 to 1 : 2. Thus, E_0' of the heme a_3^{2+}–CN in our experiment is approximately +430 mV. Note that a similar value of $E_0' = +380$ mV presented in work [17] was obtained for the heme a_3 complexed with cyanide upon addition of 5 mM KCN, whereas in our experiments cyanide was not added and could appear in the solution only as a result of dissociation of ferrocyanide, i.e. in a concentration definitely lower than 0.1 mM. Apparently, the E_0' value of heme a_3 in the cyanide complex of cytochrome ba_3 is determined not only by the ligand concentration.

The high affinity for cyanide and low rate of its binding at low concentrations did not allow us to determine the value of K_d of the cytochrome ba_3 cyanide complex by simple titration. Therefore, we tried to evaluate the complex tightness using other approaches.

First, we studied the cyanide distribution between metmyoglobin and the reduced heme a_3 of cytochrome ba_3. The affinity of metmyoglobin for cyanide at pH 7.8 is characterized by K_d = 1.2 μM [30]. Due to strict stoichiometry of the ligand binding (1 : 1), the cyanide complex of metmyoglobin can be used as a buffer system providing for a stable desired concentration of free cyanide in the range of 10^{-7}-10^{-5} M.

Figure 5a presents absolute optical absorption spectra recorded successively for oxidized metmyoglobin (spectrum *1*), for the system upon addition to it of equimolar amount of cyanide (spectrum *2*), and of the equimolar mixture (1 : 1 : 1) of metmyoglobin, cyanide, and cytochrome ba_3 (spectrum *3*). The medium also contains the weak reductant DAD (at pH 7.6 its $E_0' = +216$ mV [27]) that can reduce heme a_3 of cytochrome ba_3 (Fig. 2, a and b) but is unable to reduce noticeably metmyoglobin ($E_0' = +55$ mV [31]). Indeed, the metmyoglobin spectrum in the presence of reduced DAD (Fig. 5a, spectrum *1*) during 24 h was the same as the spectrum recorded in the absence of the reductant. Data in Fig. 5a show that upon the establishment of equilibrium in the system, the metmyoglobin-bound cyanide (spectrum *2*) is transferred completely to oxidase ba_3 (spectrum *3*). Thus, the affinity of reduced heme a_3 for cyanide is at least an order of magnitude higher than the affinity of metmyoglobin. The control experiment con-

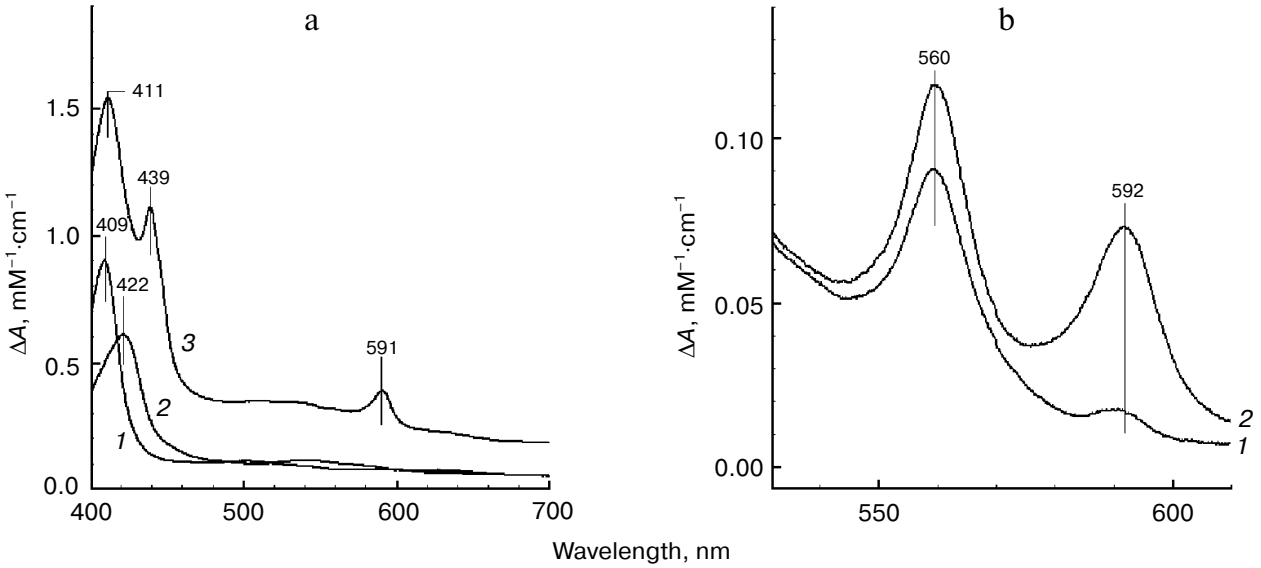

Fig. 5. Determination of tightness of the cytochrome ba_3 cyanide complex using metmyoglobin. a) Ligand redistribution between the oxidized metmyoglobin and the reduced oxidase ba_3. Successively recorded absorption spectra of 5 μM metmyoglobin (spectrum *1*), of the pair metmyoglobin/cyanide at ratio 1 : 1 (spectrum *2*), and of the system metmyoglobin/cyanide/cytochrome ba_3 at ratio 1 : 1 : 1 (spectrum *3*) are presented. The medium contains 1 mM reduced DAD. Spectrum *2* was recorded 15 min after addition of KCN, and spectrum *3* was recorded 24 h after addition of oxidase ba_3. b) Formation of oxidase ba_3 cyanide complex in the presence of ~10^{-7} M ligand. Difference absorption visible spectra (against the specimen before the cytochrome ba_3 addition) are presented. Spectra *1* and *2* were recorded, respectively, 30 min and 5 h after addition of 2 μM oxidase ba_3 to the system of metmyoglobin/cyanide (11 : 1). Other conditions are the same as in Fig. 5a.

firmed that the cyanide complex of metmyoglobin was stable for at least 48 h.

In the experiment presented in Fig. 5b, the cytochrome ba_3 was added to the cyanide-buffered system prepared by mixing metmyoglobin with cyanide at the ratio of 11 : 1 (metmyoglobin was taken in a significant molar excess relative to cytochrome oxidase). In this case, the free cyanide concentration in solution was ~10^{-7} M. As in the previous case, upon a sufficiently long incubation the cytochrome ba_3 was completely converted to the cyanide complex for which $K_d \leq 10^{-8}$ M can be estimated.

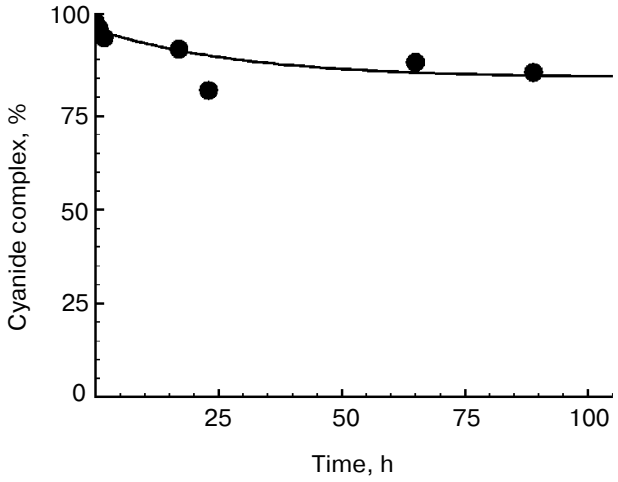

Fig. 6. Dissociation of cytochrome ba_3 cyanide complex. The ordinate axis indicates the fraction of the enzyme cyanide complex and the abscissa axis indicates the time after addition of 1 mM $ZnCl_2$ to the completely formed complex. The experiment was performed under anaerobic conditions. Zero time corresponded to the response obtained upon 60 min incubation of oxidase ba_3 in the presence of 2 mM reduced DAD and 200 μM KCN. The function $y = y_o \cdot (e^{-t/\tau})$ is plotted through experimental points, which corresponds to decomposition of ~15% of the complex with $\tau = 30$ h.

We tried to determine the rate of dissociation of the cytochrome ba_3 complex with cyanide. To preformed complex an excess of Zn^{2+} was added, which has a high affinity for cyanide (for $[Zn(CN)_4^{2-}]$, $K_d = 1.3 \cdot 10^{-17}$ M [28]). In the control experiment, addition of Zn^{2+} caused decomposition of the metmyoglobin cyanide complex. Even at great excess of Zn^{2+}, binding of cyanide with the cytochrome ba_3 was virtually irreversible (Fig. 6). The dissociation rate during the first 50 h of experiment allowed us to evaluate the $k_{off} \leq 9.3 \cdot 10^{-6}$ sec^{-1}, and no further dissociation was observed. For the dissociating fraction of the complex, the ratio k_{off}/k_{on} results in $K_d \leq 8.4 \cdot 10^{-8}$ M, which is in agreement with the data of the equilibrium experiments.

The pH dependence of formation of cytochrome oxidase ba_3 complex with cyanide. Formation of cyanide complex of ba_3-type reduced cytochrome oxidase is strongly decelerated with alkalization but without a noticeable change in the dissociation rate.

Figure 7a shows the pH dependence of cyanide binding with cytochrome ba_3: on transition from the neutral region to pH 9.5, the k_{on} value decreased sixfold. In the

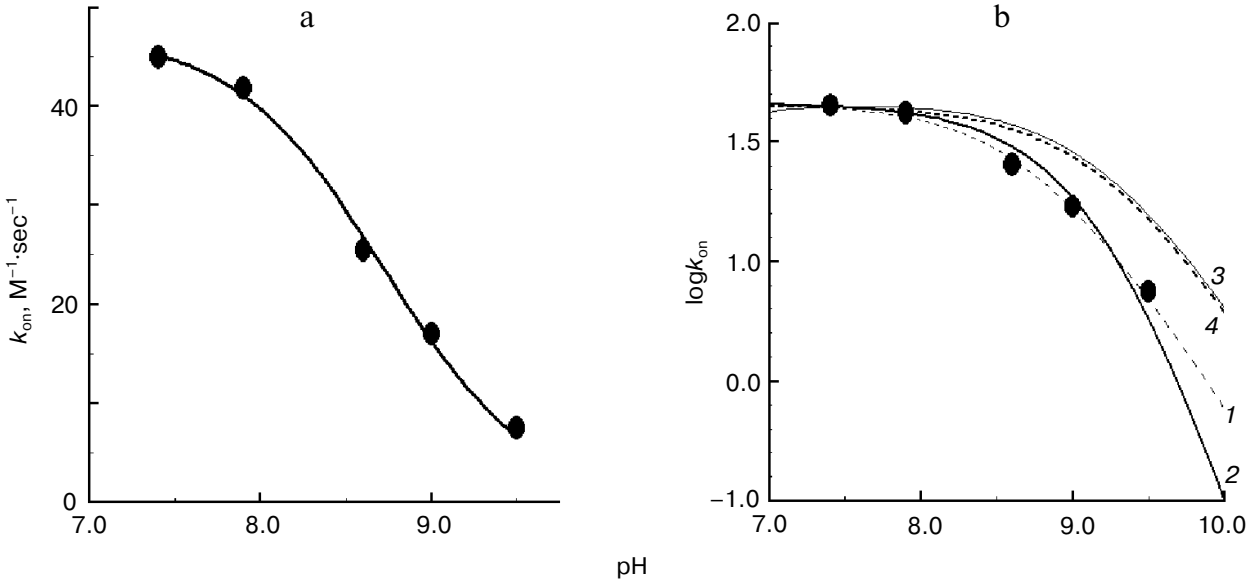

Fig. 7. The pH dependence of cytochrome ba_3 cyanide complex formation. a) The pH dependence of the cyanide-binding rate. Kinetics of the cyanide complex formation was recorded during 15 min after addition of 50 μM KCN to the enzyme preincubated for 5 min in medium with the corresponding pH value (see "Materials and Methods"). The rate constant was obtained from kinetic data using the Origin 7 program. b) Possible variants of pH-dependent interaction of the enzyme with the ligand. Data in Fig. 7a are presented on the logarithmic scale. Theoretical curves *1-4* are plotted through experimental points corresponding to the following cases of reagent interactions: *1*) protonated enzyme (pK_a = 8.75) binds any form of the ligand; *2*) protonated enzyme (pK_a = 9.28) binds the protonated ligand HCN; *3*) protonated enzyme (pK_a = 6.0) binds anionic form of the ligand CN^-; *4*) enzyme binds the protonated ligand.

alkaline region, the reaction rate becomes proportional to activity of H^+ ions (Fig. 7b). The dependence on pH disappeared in the neutral region, and the reaction rate flattens out with k_{on} = 45 $M^{-1}\cdot sec^{-1}$. The pH dependence of the cyanide binding with heme a_3 could originate in ionization of both the ligand and the enzyme. As the pK_a of HCN is 9.2, deprotonation of the ligand should inhibit the reaction rate only at pH > 9. The dependence of the process on pH under less alkaline conditions suggests the involvement in the reaction of the protonated form of the enzyme.

In Fig. 7b theoretical curves are drawn through experimental points for a number of possible variants of interaction (see the legend). Variants *3* and *4* do not agree with the experimental data. Variants *1* and *2* describe the pH dependence of the reaction rate sufficiently well, but additional experiments at pH > 9.5 are necessary to discriminate between them reliably.

DISCUSSION

Our findings confirm significant differences in chemical properties of the A-class heme–copper oxidases (cytochrome oxidases of aa_3-type from bovine heart mitochondria and from the bacteria *P. denitrificans* and *R. sphaeroides* are the best studied examples) and of the B class enzymes (of which cytochrome oxidase of ba_3-type from *T. thermophilus* is studied best). In particular, ligands can bind with the oxygen reducing center of the enzyme from *T. thermophilus* only after its reduction. This effect can be caused by at least two different factors.

1. The binuclear center in the oxidized ba_3-type oxidase is sterically closed for external ligands. Reduction of the binuclear center results in structural changes that open heme a_3 to ligands.

2. The oxygen reducing center in the oxidized ba_3-type oxidase is available for external ligands, but affinities of oxidized redox components of the center for cyanide, sulfide, azide, hydrogen peroxide, etc. are so low that at reasonable concentrations of these ligands no reactions are observed.

Conceivably, both these factors can take place.

In the three-dimensional structure of oxidized cytochrome ba_3 there is an oxygen atom between the heme a_3 iron and Cu_B [9]. This atom seems to present a doubly ionized water molecule connecting two metal cations through a μ-oxo-bridge:

$$[Fe(III)]^+\text{-}O^{2-}\text{-}[Cu(II)]^+.$$

Such a tightly bound oxygen bridge can, in particular, be a reason for the inertness of the oxidized enzyme towards external ligands. One-electron reduction of the oxygen reducing center (e.g. the transition of heme a_3^{3+} into state a_3^{2+}) has to be accompanied by protonation

of the μ-oxo-bridge and its conversion into hydroxyl anion coordinated with Cu_B^{2+} and capable of dissociation:

$$[Fe(III)]^+\text{-}O^{2-}\text{-}[Cu(II)]^+ + e^- + H^+ \rightarrow$$

$$\rightarrow [Fe(II)]^0\ {}^-HO\text{-}[Cu(II)]^+.$$

However, besides the need for binuclear center "opening" on reduction, a stable cyanide complex of cytochrome ba_3 is produced not by the oxidized Fe(III)-form, but by the reduced Fe(II)-form of heme a_3, unlike the situation with virtually all other known hemoproteins (table). In globins and peroxidases, cyanide forms a stable complex with trivalent heme iron ($K_d \sim 10^{-5}$-10^{-9} M) but virtually does not react with ferro forms of these hemoproteins ($K_d \geq 10^{-1}$ M). Although the A-class heme–copper oxidases can bind cyanide in both oxidized and reduced form, the ligand affinity for the reduced high-spin heme ($K_d \sim 10^{-4}$-10^{-3} M) in this case is also three-four orders of magnitude lower than for the oxidized heme ($K_d \sim 10^{-7}$ M). The reaction of cyanide with bivalent high-spin heme iron seems to be based mainly on making a π-acceptor bond [32], like in the case of binding of the reduced heme a_3^{2+} with carbon monoxide.

The reason of the unusually tight selective binding of cyanide with the reduced heme a_3 in cytochrome ba_3 is unclear (unfortunately, the three-dimensional structure of the oxidase ba_3 complex with cyanide has not been published yet). On binding with the oxidized enzyme in the A-class oxidases, cyanide forms a bridge between the iron (III) of the heme a_3 and Cu_B(II) ion of the "invisible" copper [33]. Formation of such a bridge in the binuclear center of the oxidized cytochrome ba_3 can be hindered because of the smaller distance between the heme a_3 and Cu_B than in the bovine enzyme (4.7 vs. 4.9 Å) [15, 34]. Sterically-induced distortion of the angle of the Fe–C–N valence bond can destabilize the cyanide complex with the oxidized form of the enzyme. Moreover, as discussed in work [32], the interaction between Fe(II) of the heme a_3 with the axial histidine is markedly weaker in ba_3-type cytochrome oxidase than in the A-class aa_3-type oxidases, and this should be accompanied by increased tightness of the interaction of the reduced heme with cyanide at the cost of displacement of electron density of the corresponding σ-bond from the histidine nitrogen towards the iron atom. In fact, in the same work the oscillation frequency of the Fe(II)–CN bond in the cyanide ba_3 complex is shown to be abnormally high (512 vs. 469 cm^{-1} in the cyanide complex of reduced aa_3 oxidase). These data are consistent with the three-dimensional structures of the two enzymes: in cytochrome ba_3 the distance between the heme a_3 iron and nitrogen of the proximal histidine is unusually high (2.5-3.3 [9, 14] vs. 1.9-2.1 Å in the class A-class oxidases from *P. denitrificans* [7] or *R. sphaeroides* [13]). Moreover, in work [32] a linear arrangement of the Fe–C–N atoms is shown for the cyanide complex of cytochrome ba_3, whereas in the reduced aa_3 oxidase the corresponding bonds form an angle of 170° (this has been confirmed by data on the three-dimensional structure of the cyanide complex of the reduced cytochrome oxidase from *R. sphaeroides* [35]). In the case of ba_3 oxidase, an unstrained linear structure of Fe^{2+}–C–N should correspond to the more stable complex.

Even the very affinity value of the reduced cytochrome ba_3 for cyanide is unusual. In particular, cytochrome ba_3 can extract cyanide from ferrocyanide (Fig. 4a), which must be taken into account in experiments with $K_4Fe(CN)_6$ used as a reductant. Unfortunately, a relatively slow binding of cyanide combined with a very high affinity for the heme a_3^{2+} makes difficult the quantitative evaluation of K_d. In the present

Characteristics of the interaction of cyanide with various hemoproteins

Hemoproteins	K_d, M	k_{on}, $M^{-1}\cdot sec^{-1}$	k_{off}, sec^{-1}	Source	K_d, M	k_{on}, $M^{-1}\cdot sec^{-1}$	k_{off}, sec^{-1}	Source
	Oxidized form				Reduced form			
ba_3 cytochrome oxidase from *T. thermophilus*	does not bind			[18], this study	$\leqslant 8.4\cdot10^{-8}$	$1.11\cdot10^2$	$\leqslant 9.3\cdot10^{-6}$	this study
aa_3 cytochrome oxidase from bovine heart	$\sim10^{-6}$	~2	$\sim2\cdot10^{-6}$	[43]	$0.23\cdot10^{-3}$ $0.35\cdot10^{-3}$	$2.35\cdot10^2$	$\sim6\cdot10^{-2}$	[24], this study
Sperm whale myoglobin	$1.2\cdot10^{-6}$	$2.0\cdot10^2$	$2.4\cdot10^{-4}$	[30]	0.4	–	$2.1\cdot10^{-2}$	[32]
Human hemoglobin	$1.3\cdot10^{-9}$	$1.1\cdot10^2$	$1.4\cdot10^{-7}$	[32]	~1	–	$1.2\cdot10^{-1}$ (R) 1.5 (T)	[32]
Horseradish peroxidase	$\sim2.5\cdot10^{-6}$	$9.0\cdot10^4$	$2.8\cdot10^{-1}$	[32]	$\sim7\cdot10^{-4}$	29	$2.5\cdot10^{-2}$	[32]
Hemoglobin P from *Campylobacter jejuni*	$5.8\cdot10^{-9}$	$\geqslant2\cdot10^4$	$\geqslant1\cdot10^{-4}$	[32]	$1.2\cdot10^{-6}$	$3.3\cdot10^3$	$4.0\cdot10^{-3}$	[32]

work we have obtained only the upper value for K_d of the cytochrome ba_3 complex with cyanide: according to both kinetic and equilibrium experiments, it does not exceed 10^{-8} M.

It is not excluded that, unlike the aa_3-type reduced oxidase which forms a cyanide complex with the stoichiometry of 1 : 1 [35], the cytochrome ba_3 binuclear center can simultaneously bind two cyanide molecules as the ligands of the reduced heme a_3 and the oxidized copper Cu_B, respectively [36]. In this case, the affinity of the reduced heme a_3 for cyanide might depend on the redox state of the "invisible" Cu_B. In the cyanide complex of the dithionite-reduced aa_3 oxidase the binuclear center is fully reduced; however, experimental conditions in most works with ba_3 oxidase cyanide complex allow for the one-electron reduction of the binuclear center with production of a structure of a_3^{2+}–CN, Cu_B^{2+}–CN. The second cyanide molecule coordinated with Cu_B(II) is likely to "lock" the heme a_3^{2+} ligand in the active site, and this makes the reaction virtually irreversible. Unfortunately, the data on binding of the second cyanide molecule with Cu_B(II) in ba_3-type oxidase are indirect [36] and need yet to be confirmed by analysis of the three-dimensional structure. Note also that a successive reduction of cytochrome ba_3 redox centers, including Cu_B, does not influence the tightness of the heme iron bond with cyanide [32, 37].

We have no data on the Cu_B redox state in the cyanide complex produced under our conditions. In most experiments, DAD was used as a reductant ($E_0' = +216$ mV at pH 7.6 [26]), which can easily reduce the binuclear center of the cytochrome ba_3 upon the exhaustion of oxygen, but, unlike dithionite, does not remove oxygen from the solution. If Cu_B retains the ability for autooxidation upon the formation of the reduced heme a_3 cyanide complex [17], the structure of the cytochrome ba_3 cyanide complex in our experiments can be similar to the structure (a_3^{2+}–CN, Cu_B^{2+}–CN) described in work [36]. It would be interesting to compare the affinity of cytochrome ba_3 for cyanide in the cases of one- and two-electron reduction of its binuclear center. It is not excluded that reduction of Cu_B can lead to a significant decrease in the tightness of the cyanide complex, the K_d value approaching those determined for the reduced A-class oxidases (~10^{-4} M).

The pH dependence of cyanide binding was studied in detail for the oxidized form of mitochondrial cytochrome oxidase [38-42], but we are not aware of similar studies for the reduced enzyme. In the case of oxidized cytochrome oxidase, HCN was shown to interact with the protonated enzyme (pK_a of the group which controls the ligand binding varied from 6.5 to 7.2 in the enzyme from different sources). In the present work, we also find that alkalinization results in a decrease in the rate of the reduced cytochrome ba_3 binding with cyanide, but pK_a of the group whose deprotonation decelerates the ligand binding is significantly higher (8.75) (Fig. 7).

The rate of the cytochrome ba_3 cyanide complex formation is more likely to be proportional to concentration of the protonated ligand, HCN. The necessity of protonation of the ligand can be associated with the way of cyanide entrance into the oxygen reducing center. According to the data on the three-dimensional structure of heme–copper oxidases, the binuclear center is located deep in the protein and is screened from contact with substances dissolved in water. Proton channels leading to the active center can pass water molecules but seem to be impermeable for compounds containing two and more "heavy" atoms. Therefore, attention should be paid to the oxygen channel leading from the oxygen-enriched hydrophobic lipid bilayer of the membrane to the binuclear center. The oxygen channel is especially distinct in the three-dimensional structure of ba_3-type cytochrome oxidase; it is wider than in the canonical oxidases, and is Y-shaped where it opens into the membrane [9, 15]. This channel may serve for entrance into the binuclear center of two- and three-atom ligands, such as molecular oxygen and its antagonists (CO, NO). The oxygen channel is very hydrophobic; therefore, even compounds which bind with the heme iron as anions (cyanide, azide, sulfide) are likely to enter the site of the reaction in an uncharged protonated form.

The features specific for interaction of cytochrome ba_3 from *T. thermophilus* with ligands and, in particular, with cyanide, can be also characteristic for other B-group heme–copper oxidases. According to our preliminary data (unpublished results of the work in cooperation with the laboratory of M. Teixeira, Institute of Chemical and Biological Technology, New Lisbon University), another B-class oxidase, quinol oxidase of aa_3-type from *Acidianus ambivalens*, binds ligands of the oxygen reducing center also only upon reduction, and the cyanide complex is very tight.

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