Fourier-transform infrared studies on azide-binding to the binuclear center of the *Escherichia coli bo*-type ubiquinol oxidase[1]

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Abstract Azide-binding to the heme-copper binuclear center of *bo*-type ubiquinol oxidase from *Escherichia coli* was investigated with Fourier-transform infrared spectroscopy. Deconvolution analyses of infrared spectra of the azide ($^{14}N_3$)-inhibited air-oxidized form showed a major infrared azide antisymmetric stretching band at 2041 cm^{-1}. An additional band developed at 2062.5 cm^{-1} during a longer incubation. Isotope substitutions with terminally ^{15}N-labelled azides did not show a splitting of the major band, indicating that the geometry of the bound azide is mainly in a bridging configuration between high-spin heme *o* and Cu_B. The band at 2062.5 cm^{-1} showed clear splittings upon substitution with the terminally ^{15}N-labelled azides, indicating the Cu_B^{2+}-N = N = N structure. Partial reduction of the oxidase with β-NADH in the presence of azide caused an appearance of new infrared bands at 2038.5 (major) and 2009 (minor) cm^{-1}. The former band also showed clear splittings in the presence of the terminally ^{15}N-labelled azides, indicating that reduction of low-spin heme *b* alters the structure of the binuclear center leading to the Fe_o^{3+}-N = N = N configuration.

Key words: *Escherichia coli bo*-type ubiquinol oxidase; Fourier-transform infrared; Heme-copper binuclear center; Azide; Isotope label; Bridging structure

1. Introduction

Cytochrome *bo*-type ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli* is a member of the heme-copper terminal oxidases and catalyzes the two electron oxidation of ubiquinol-8 and the four electron reduction of dioxygen to water [1,2]. Redox reactions mechanistically couple with the formation of an electrochemical proton gradient across the cytoplasmic membrane not only by scalar protolytic reactions at the inner and outer surfaces of the membrane but also by a proton pumping mechanism [1,2]. It is believed that the last two steps of the four electron transfer reactions to dioxygen are linked to the proton pumping by cytochrome *c* oxidase [3]. Thus, it becomes increasingly important to analyze the mixed valence states of the oxidase as a model for the intermediate species of the dioxygen reduction chemistry, since the understanding of the redox-linked structural change(s) at the metal center appears to be a key issue to reveal the redox-linked proton pumping.

In a previous Fourier-transform infrared (FTIR) study, Tsubaki analyzed azide-binding to the Fe_{a3}-Cu_B binuclear center of bovine heart cytochrome *c* oxidase [4]. In the azide-inhibited air-oxidized form, a bound azide showed a major infrared band at 2051 cm^{-1}, assignable to a bridging structure, Fe_{a3}^{3+}-N = N = N-Cu_B^{2+}. Recent, high resolution X-ray structure of cytochrome *c* oxidases in the azide-inhibited air-oxidized form [5,6] confirmed the azide-bridging structure. As the reduction level of the four metal centers increases, the azide-inhibited oxidase showed three kinds of bound azide infrared bands assignable to the Fe_{a3}^{3+}-N = N = N structures, suggesting that redox-linked conformational changes occur at the binuclear center [4]. Subsequently, Tsubaki et al. carried out a combined EPR and FTIR spectroscopic study on the *E. coli bo*-type ubiquinol oxidase [7]. The azide-inhibited air-oxidized form showed an EPR signal from an integer spin system confirming the existence of the spin-spin exchange-coupled binuclear site [7]. The azide bound to the air-oxidized enzyme exhibits an infrared band at 2041 cm^{-1}, characteristic to the bridging structure [7]. In the present study, we extended the FTIR study on the *E. coli bo*-type ubiquinol oxidase further to clarify the redox-linked conformational change at the heme-copper binuclear center using azide as a monitoring probe.

2. Materials and methods

2.1. Purification of the bo-type ubiquinol oxidase

The enzyme was purified from the *E. coli* cytoplasmic membranes as previously described [7].

2.2. Measurement of FTIR and optical spectra

FTIR spectra of the enzyme were measured at 10°C with a Perkin-Elmer FTIR spectrophotometer (model 1850) with a nominal spectral resolution of 4.0 cm^{-1} [7]. The spectra were analyzed with a deconvolution technique as previously described [4]. Absolute optical spectra of the enzyme in the infrared cells were recorded at room temperature before and after FTIR measurements. Partially reduced azide-inhibited enzymes were prepared according to Yoshikawa and Caughey using β-NADH and phenazine methosulfate [8] with slight modifications as described previously [4]. The following sodium azide isotopes were used: $Na^{14}N_3$ (natural abundance, Nacalai Tesque, Kyoto, Japan), $Na^{15}N^{14}N^{14}N$ (99 atom % ^{15}N, ICON, Mt. Marion, NY, USA), $Na^{15}N^{15}N^{14}N$ (95 atom % ^{15}N, Berlin Chemie, Berlin, Germany) and $Na^{15}N_3$ (95 atom % ^{15}N, Laboratorien Berlin-Adlers-

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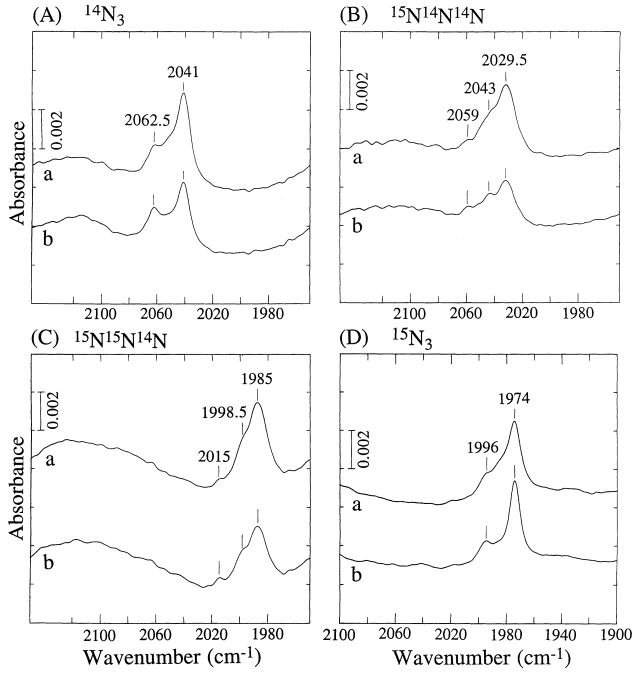

Fig. 1. Bound azide infrared spectra of the *E. coli bo*-type ubiquinol oxidase in the air-oxidized state. (A) $^{14}N_3$, (B) $^{15}N^{14}N^{14}N$, (C) $^{15}N^{15}N^{14}N$, (D) $^{15}N_3$. In each panel, the upper line (a) represents the spectrum just after addition of azide, whereas the lower line (b) shows the spectrum one day after addition of azide. Experimental conditions were: sample concentration, 0.45 mM; azide concentration, 0.8 mM; temperature, 10°C; spectral data accumulation, 400 cycles (2 h); nominal spectral resolution, 4.0 cm^{-1}.

hof GmbH, Berlin, Germany). Other chemicals were commercial products of analytical grade.

3. Results

Addition of a slight excess amount of $^{14}N_3$ to the air-oxidized enzyme showed a major infrared azide band at 2041 cm^{-1} (Fig. 1Aa, Table 1). Substitution with terminally ^{15}N-labelled azides ($^{15}N^{14}N^{14}N$ or $^{15}N^{15}N^{14}N$) did not result in a splitting of this band (Fig. 1Ba, Ca, Table 1), as previously observed for cytochrome *c* oxidase in the air-oxidized state [4,8]. A new band at 2062.5 cm^{-1} developed during the incubation of the sample in the infrared cell at 4°C (Fig. 1Ab). Upon azide isotope substitution with $^{15}N^{14}N^{14}N$ or $^{15}N^{15}N^{14}N$, this mode showed splittings of about 16 cm^{-1} (Fig. 1Bb, Cb, Table 1). Substitution with $^{15}N_3$ caused no splitting (Fig. 1), as expected. The development of the band intensity was not accompanied by the reduction of the heme moiety, on the basis of the visible absorption spectra. Deconvolution analyses of the azide infrared bands confirmed the presence of two azide species, i.e. the 2041 cm^{-1} (species a) and the 2062.5 cm^{-1} species (species b) in addition to free azide ions (species e). Addition of cyanide (final 10 mM) to the azide ($^{14}N_3$)-inhibited air-oxidized enzyme caused the disappearance of both azide bands at 2041 and 2062.5 cm^{-1}, simultaneously, leaving the free azide band at 2049 cm^{-1} and the bridging cyanide band at 2146 cm^{-1} [7].

Partial reduction (2/3-reduced) of the metal centers of the oxidase with β-NADH in the presence of azide ($^{14}N_3$) showed a new sharp azide infrared band at 2038.5 cm^{-1} (Fig. 2a). Additionally, a weak band appeared at 2009 cm^{-1} (Fig. 2a). Although the frequency of the former band, which was overlapped with the band derived from the free azide ion, is very close to that of the major 2041 cm^{-1} band of the azide-inhibited air-oxidized state, the 2038.5 cm^{-1} species (species c) is structurally distinct from the 2041 cm^{-1} species (species a) since the corresponding mode in the presence of terminally-labelled azides ($^{15}N^{14}N^{14}N$ or $^{15}N^{15}N^{14}N$) showed a clear doublet at 2032 and 2021.5 cm^{-1} or at 1987.5 and 1977 cm^{-1}, respectively (Fig. 2b, c, Table 1). However, substitution with $^{15}N_3$ showed only one band at 1972 cm^{-1} (Fig. 2d), as expected. The splitting of the minor 2009 cm^{-1} species (species d) in the presence of terminally-labelled azides ($^{15}N^{14}N^{14}N$ or $^{15}N^{15}N^{14}N$) was not confirmed due to its weak intensity in absorbance. The appearance of both the 2038.5 and 2009 cm^{-1} bands was accompanied by the reduction of low-spin heme *b* based on the visible absorption spectra (i.e. increase in absorbance of the α-band at 561 nm). Changes in the reduction level of the metal centers (from 1/3- to 3/3-reduced) with β-NADH did not produce any other bound azide species in the infrared spectra.

4. Discussion

4.1. Azide-inhibited air-oxidized state

There are several factors which can affect the azide antisymmetric stretching frequency [9]. The most fundamental is a change in the metal-N(azide) bond order. Indeed, the difference in ν_{max} between the high-spin and low-spin azide band was explained by an increase of the Fe^{3+}-N(azide) bond order (concomitant with a decrease in the bond order of the intra-azide bonds) [10]. Thus, low-spin heme-azide species gives a band around 2020 cm^{-1} whereas the high-spin heme-azide species shows it around 2045 cm^{-1} [9,10]. For the air-oxidized

Table 1
Band assignments of azide antisymmetric stretching vibrations bound to the Fe_o-Cu_B binuclear center of the *E. coli bo*-type ubiquinol oxidase

Species	$^{14}N_3$	$^{15}N^{14}N^{14}N$	$^{15}N^{15}N^{14}N$	$^{15}N_3$	Assignment
a	2041	2029	1985	1974	Fe_o^{3+}-N = N = N-Cu_B^{2+} ($g^* = 3.2$)
b	2062.5	2043	1999	1996	Cu_B^{2+}-N = N = N
		2059	2015		
c	2038.5	2032	1988	1972	Fe_o^{3+}-N = N = N (high-spin)
		2021.5	1977.5		
d	2009	ND	ND	1943	Fe_o^{3+}-N = N = N (low-spin; $g_z = 2.89$)
e	2049	2038	1991	1982	Free azide
Cu-DETA-$N_3^{(f)}$	2068				Cu^{2+}-N = N = N
$Cu^{2+}Zn^{2+}SOD^{(f)}$	2058				Cu^{2+}-N = N = N

(f) Leone et al. (1998) [15]. DETA, diethylenetriamine; SOD, superoxide dismutase.

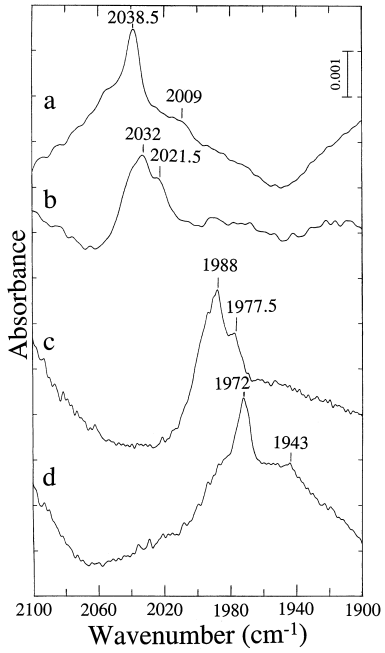

Fig. 2. Bound azide infrared spectra of the *E. coli bo*-type ubiquinol oxidase in the 2/3-reduced state in the presence of $^{14}N_3$ (a), $^{15}N^{14}N^{14}N$ (b), $^{15}N^{15}N^{14}N$(c) and $^{15}N_3$ (d). Experimental conditions were the same as in Fig. 1, except for the enzyme concentration (0.5 mM) and azide concentration (0.45 mM). The /nNominal spectral resolution for the spectra of $^{15}N^{15}N^{14}N$ and $^{15}N_3$ derivatives was 2.0 cm^{-1}.

E. coli bo-type ubiquinol oxidase in solution state, the bound azide gave a major infrared band at 2041 cm^{-1} (for $^{14}N_3$). Since the binding site of azide is considered to be ferric heme *o*, the 2041 cm^{-1} species (species a) can be classified as a heme *o*-azide derivative in the high-spin state. Substitutions with asymmetrically ^{15}N-labelled azides ($^{15}N^{14}N^{14}N$ and $^{15}N^{15}N^{14}N$), however, did not cause a splitting of the infrared band. Further, we found that a major bound azide band at 2041 cm^{-1} of the *Acetobacter aceti* ubiquinol oxidase did not cause a splitting upon the ^{15}N isotope substitutions for both *ba*- and *bo*-type enzymes in the air-oxidized state [11]. These properties are very similar to the corresponding band at 2051 cm^{-1} of the azide-inhibited air-oxidized bovine cytochrome *c* oxidase [4,8]. These unusual properties were the basis of our original proposal of the azide-bridging configuration at the heme-copper binuclear center (i.e. Fe_o^{3+}-N = N = N-Cu_B^{2+} structure) [4]. Indeed, heme-azide complexes of ferri-myoglobin and hemoglobin showed splittings of $\sim$12 cm^{-1} for high-spin, $\sim$16 cm^{-1} for low-spin species upon substitution with $^{15}N^{14}N^{14}N$ or $^{15}N^{15}N^{14}N$ (e.g. 12.7 cm^{-1} for high- and 16.1 cm^{-1} for low-spin states, respectively, of the $^{15}N^{14}N^{14}N$ complex and 13.8 cm^{-1} for high- and 17.5 cm^{-1} for low-spin states, respectively, of the $^{15}N^{15}N^{14}N$ complex of horse skeletal muscle myoglobin). Previously, Pate et al. have examined the ^{15}N isotope splitting in a series of model copper complexes and shown that the splitting obtained with $^{15}N^{14}N^{14}N$ is between 6 and 11 cm^{-1} for a terminally bound azide (Cu-N-N-N), <2 cm^{-1} for μ-1,3-bridged azide derivatives (Cu-N-N-N-Cu) and greater than 17 cm^{-1} for μ-1,1-bridged derivatives [12].

These infrared results are consistent with recent high-resolution X-ray crystal structures of the azide-inhibited air-oxidized cytochrome *c* oxidases, which showed the presence of a clear electron density, most likely an azide ion, between Fe_{a3} and Cu_B [5,6]. Thus, it can be concluded that the major azide bound species of the air-oxidized heme-copper terminal oxidases adopts a bridging configuration with an Fe^{3+}-N = N = N-Cu_B^{2+} structure for both in solution and in crystalline states.

In the azide-inhibited air-oxidized form of bovine cytochrome *c* oxidase, EPR signals from the heme-copper binuclear site were not so much different from those of the air-oxidized form [4]. On the other hand, for the azide-inhibited *E. coli* [7] and *A. aceti* [11] ubiquinol oxidases, an EPR signal ($g^* = 3.2$) derived from an integer spin system produced by a spin-spin exchange coupling at the binuclear site appeared. Although the comparison of the coupling efficiency via a bridging azide is difficult to estimate, it is clear that the geometry of the azide-bridging might be different from each other. The clear difference in the peak positions of the major bound azide band between bovine cytochrome *c* oxidase (2051 cm^{-1}) and the ubiquinol oxidases (2041 cm^{-1}) suggests such distinctions. Indeed, a similar difference was previously observed for the bridging cyanide stretching vibrations (2152 versus 2146 cm^{-1}, respectively) [7,11,13] and was explained due to the difference in the Cu_B-N-C bond angle and Cu_B-N bond distance [14].

4.2. The 2062.5 cm^{-1} band

The 2062.5 cm^{-1} band (species b) had no counterpart in the infrared spectra of the azide-inhibited air-oxidized form of cytochrome *c* oxidase. The unusually high frequency suggests that this is neither due to the high-spin nor low-spin heme-azide species. The 16 cm^{-1} splittings upon substitution with terminally ^{15}N-labelled azide indicates that the azide ion binds to a metal center not in a bridging but in an end-on configuration. Further, this band (as well as the 2041 cm^{-1} band) disappeared completely upon the addition of cyanide to the pre-formed azide-inhibited species leading to a formation of the bridging cyanide band at 2146 cm^{-1} [7]. We, therefore, propose that the 2062.5 cm^{-1} band is likely due to a Cu_B^{2+}-N = N = N species. Being consistent with this assignment, the Cu^{2+}-diethylenetriamine-azide model compound and Cu^{2+}, Zn^{2+}-superoxide dismutase-azide complex showed copper bound azide infrared bands at 2068 and 2058 cm^{-1}, respectively (Table 1) [15].

One difficulty for the assignment of the Cu_B^{2+}-N = N = N species is its unusually high binding affinity. With model complexes in aqueous solvents, the binding affinity is weak with dissociation constants ranging from 20 mM for Cu to 7 mM for Cu^{2+}. These values decrease markedly with a decrease in dielectric and values less than 0.2 mM are found for Cu^{2+} in methanol [16]. Indeed, the azide-bindings to copper proteins show large variations. However, when azide binds to a copper protein as a terminal ligand, the dissociation constant is usually much higher than 1 mM ($K_d = 7.2 \sim 11.4$ mM for Cu, Zn-superoxide dismutase [17,18], 7.5 mM for diamine oxidase [19], 23.8 mM for amine oxidase [20], 10 mM for ascorbate oxidase [21]). Some copper proteins carrying a type 3 site are known to have a relatively high binding affinity towards azide (2 mM for molluscs hemocyanin [22], 0.33 mM for *Neurospora* tyrosinase [23]), but their coordinations are believed to adopt in a bridging configuration between two copper atoms

in either a μ-1,3 or μ-1,1 geometry [12,24]. The heme *o* bound water ligand or amino acid residue(s) in close proximity may have some role to stabilize the Cu_B^{2+}-N = N = N structure.

Previously, Little et al. proposed that azide binds to a pulsed form of *bo*-type ubiquinol oxidase with a terminal ligand to Cu_B^{2+} in a stoichiometry of 1:1, yielding a binuclear center in the form of Fe^{3+}-OH_2::Cu_B^{2+}-N_3 [25]. The cause of the discrepancy is not clear at this stage but may be due to a structural difference between the as prepared air-oxidized form and the pulsed form.

4.3. Partially reduced azide-inhibited states

It is of particular interest to note that, in the partially reduced azide-inhibited states, very similar EPR species exist at a low temperature in both cytochrome *c* oxidase and *bo*-type ubiquinol oxidase. Two low-spin EPR species have been detected for *bo*-type ubiquinol oxidase: one with $g_z = 2.79$, $g_y = 2.18$, $g_x = 1.74$ (for the Cu_B-reduced and heme *b*-oxidized species) [7,26] and the other with $g_z = 2.89$, $g_y = 2.20$, $g_x = 1.68$ (for both the Cu_B- and heme *b*-reduced species) [26]. Corresponding EPR signals of bovine cytochrome *c* oxidase have been observed at $g_z = 2.77$, $g_y = 2.18$, $g_x = 1.74$ (for the Cu_B-reduced and heme *a*-oxidized species) and at $g_z = 2.88$, $g_y = 2.19$, $g_x = 1.64$ (for both the Cu_B- and heme *a*-reduced species), respectively [27]. Resemblance of the EPR signals suggests that the crystal field parameters of the Fe^{3+}-N_3 moiety and the strength of the Fe^{3+}-N(azide) bond are virtually identical in each corresponding species at a low temperature (15 K).

For the partially reduced azide-inhibited bovine cytochrome *c* oxidase, we observed three kinds of bound azide species in infrared spectra (at 2003.5, 2015.5 and 2040 cm^{-1}), depending on the reduction level of the metal centers, each assignable to a Fe_{a3}^{3+}-N = N = N structure [4]. For the partially reduced azide-inhibited *bo*-type ubiquinol oxidase, however, we observed only two bands, i.e. the 2038.5 cm^{-1} band (species c) and the minor 2009 cm^{-1} band (species d).

The 2038.5 cm^{-1} band could be classified as a high-spin heme-azide species, whereas the minor 2009 cm^{-1} band may be derived from a low-spin heme-azide species. The 10.5 cm^{-1} splitting of the former mode upon substitution with terminally ^{15}N-labelled azide seems to support this view, since the splittings are relatively small for the high-spin species, as described above. In partially reduced forms of bovine cytochrome *c* oxidase (where both Cu_B and heme *a* are in the reduced state), the 2040 cm^{-1} high-spin species and the 2015.5 cm^{-1} low-spin species coexist [4] and may be in a thermal spin equilibrium with the low-spin state as a dominant species at room temperature. The 2040 cm^{-1} species, which showed a 11 cm^{-1} splitting upon substitution with terminally ^{15}N-labelled azide [4], is likely to be a corresponding counterpart of the 2038.5 cm^{-1} species. However, for the *E. coli bo*-type ubiquinol oxidase (where both Cu_B and heme *b* are in the reduced state), there seems no such a thermal spin equilibrium. The 2038.5 cm^{-1} high-spin species dominates at room temperature. Upon freezing or lowering the temperature to 15 K, the low-spin species becomes now detectable as the $g_z = 2.88$ EPR signal. We could not detect an infrared band corresponding to the 2003.5 cm^{-1} species of bovine cytochrome *c* oxidase, which should give the $g_z = 2.79$ EPR signal at the low temperature. For the azide-inhibited *E. coli bo*-type ubiquinol oxidase, the state with the Cu_B reduced and heme *b* oxidized may be not so stable at room temperature.

It is very likely that electrostatic interactions between the heme bound azide and Cu_B ion, three imidazole rings from the conserved His residues (His-284, His-333, His-334) and one phenol ring from Tyr-288, are major factors controlling the ν_{max} of the azide antisymmetric stretching frequency. A direct interaction (via a hydrogen bond) of heme bound azide with one of those side chains is also highly likely. Probably, there are several potential minima for which the heme bound azide can adopt with different Fe^{3+}-N_1-N_2 bond angles and exhibit the different ν_{max} value in each redox level. Even a breakage of one of the coordination bonds from the conserved imidazole groups to the Cu_B center may occur, as suggested for cytochrome *c* oxidase from the soil bacterium *Paracoccus denitrificans* [5]. If this is the case, this difference suggests an important distinction between cytochrome *c* oxidase and *bo*-type ubiquinol oxidase. This may also be related to distinctions of the electron-donating substrates, cytochrome *c* (a hydrophilic one electron carrier) and ubiquinol (a hydrophobic two electron and two proton carrier) and, furthermore, to the mechanism of the proton pumping between these two enzymes.

In conclusion, the infrared absorption bands of the coordinated azide ion can detect the changes of the local environment around the binuclear center in a great sensitivity and, therefore, is particularly useful to probe the structural changes caused by reduction of the metal centers which may be directly coupled to the proton pumping. Further studies are still in progress to reveal the nature of the redox-linked conformational changes around the heme-copper binuclear center.

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