Hydrogen Bonding in the Blue-Copper Site. Resonance Raman Study

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The blue-copper proteins azurin from *Pseudomonas aeruginosa*, azurin from *Alcaligenes denitrificans*, and the mutant Met121Gln of azurin from *Alcaligenes denitrificans* have been investigated by resonance Raman spectroscopy. Large deuterium isotope shifts up to 6 cm^{-1} have been observed for bands in the 350−460 cm^{-1} range after incubation of the apoproteins in D_2O and subsequent reconstitution with copper. The shifts derive from the deuteration of the amide hydrogens of Asn47 and Phe114 that form a hydrogen bond with the sulfur of the copper-coordinated cysteine. This observation reveals the coupling of the copper−sulfur and sulfur−hydrogen vibrations and indicates that these amide groups have to be taken into account to properly describe the electronic structure of the blue-copper site.

Introduction

High-resolution X-ray crystallography on blue-copper proteins[1−5] has revealed a largely conserved metal coordination, in which the copper is ligated equatorially by the nitrogens of two histidines and the sulfur of a cysteine. Variations occur as regards the nature of the axial ligand and the structural arrangement of the ligands around copper, which appear to be connected to differences in the electronic properties of the redox-active copper site. In this respect, also the hydrogen bonds to the sulfur of the cysteine may play a role.[2] For plastocyanin from *Populus nigra*, the sulfur of Cys84 has been proposed to be hydrogen bonded to the backbone amide proton of Asn38,[3] while for azurin the sulfur of Cys112 has been proposed to be involved in two hydrogen bonds to the backbone protons of Asn47 and Phe114.[2] Direct evidence for a hydrogen bond to the cysteine sulfur in azurin came from NMR spectra of cobalt-substituted azurin[6] and of azurin itself,[7] which reveal a small amount of spin density on the amide proton of Asn47. Recently, Freeman discussed the importance of the hydrogen bonds at the sulfur in relation to conformational differences in the copper-binding side chains.[8] The present paper concerns a study by resonance Raman spectroscopy of the hydrogen bonds of the cysteine sulfur for *Pseudomonas aeruginosa* (*P. aeruginosa*) azurin, *Alcaligenes denitrificans* (*A. denitrificans*) azurin, and the *A. denitrificans* azurin mutant M121Q.

The absorptions around 450 and 600 nm, characteristic for blue-copper proteins, concern transitions in which an electron is promoted from an orbital that is bonding to an orbital that is antibonding between copper and sulfur. When excitation light in this wavelength region is used, the Raman spectrum reveals a series of resonance-enhanced bands between 350 and 460 cm^{-1}.[9−11] The intensity of these bands derives from normal modes that are a mixture of the enhanced copper−sulfur stretch vibration and intraligand vibrations.[12] The resonance Raman spectra of various blue-copper proteins show significant differences, which are related to variations in the electronic and geometric structures of the copper sites. The frequencies of the intense bands have been reported to correlate with the strength of the bond between copper and sulfur, and with the ratio of the extinction coefficients of the absorption bands at 450 and 600 nm.[13] Recently, a resonance Raman study has been performed on plastocyanin and plastocyanin mutants with variable capability to form a hydrogen bond to the sulfur of the cysteine.[14] By mutating the Asn38, this study demonstrates that the resonance Raman spectrum of plastocyanin is sensitive to changes in the hydrogen-bonding network around the sulfur, and that the strength of the bond between copper and sulfur is modulated by the hydrogen bond of the amide proton of Asn38 to the sulfur.

Upon $^{63}Cu/^{65}Cu$[12,15,16] and $^{32}S/^{34}S$[16,17] substitution, shifts of about 1 and 3 cm^{-1}, respectively, have been observed for the bands between 350 and 460 cm^{-1} in the Raman spectrum of blue-copper proteins. These shifts confirm the copper−sulfur stretch character of the bands. Besides, isotope shifts of about 1 cm^{-1} have been found upon incubation of the proteins in D_2O. Their origin is under debate, and the shifts are considered to derive either from deuteration of the copper-coordinated histidines[12,15] or from deuteration of the amide protons that form a hydrogen bond with the sulfur of the cysteine.[9,18,19] For *P. aeruginosa* azurin, according to nuclear magnetic resonance (NMR) spectroscopy, a substantial number of amide protons does not exchange when the sample is incubated in D_2O, whereas they do exchange when the apoprotein is incubated in D_2O.[20] A complete sequential assignment of the proton signals in the NMR spectrum[21] showed that the amide protons of Asn47 and Phe114 are among those that only exchange for the apoprotein. In the present experiments we therefore started from the apoproteins in solution, exchanged the solution to D_2O, and then reconstituted the proteins with copper. We will refer to such samples as azurin reconstituted in D_2O.

For all azurins, when reconstituted in D_2O, we observe deuterium isotope shifts of bands in the resonance Raman spectrum that are large (up to 5.9 cm^{-1}) compared to those

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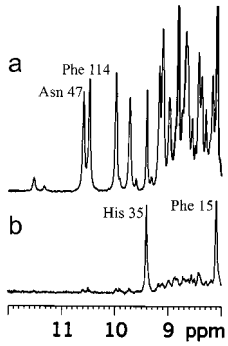

Figure 1. Proton NMR spectra at 600 MHz for wild type *P. aeruginosa* azurin in D_2O (a) and reconstituted azurin in D_2O (b).

observed for native proteins in D_2O. These shifts derive from the exchange of the amide protons that form hydrogen bonds with the copper-coordinated sulfur of the cysteine.

Materials and Methods

The azurins were purified according to procedures reported previously,[4,22−24] and the copper was removed according to the protocol described in ref 22. The solution of the apoprotein was exchanged to D_2O and subsequently incubated for 7 days at $T = 40$ °C and pH = 7 before it was concentrated. To reconstitute the protein, 1.2 equiv of Cu(II), in the form of $Cu(NO_3)_2$, was added to the solution. The final concentration of the protein was about 1 mM and the pH was 8.5.

Proton NMR spectra were recorded at 40 °C on a Bruker DMX 600 MHz NMR spectrometer with standard Bruker pulse programs. Potassium chloride was added to the *P. aeruginosa* azurin samples used in the NMR experiment to an end concentration of 25 mM. The pH values were not corrected for the deuterium effect. In total, 128 scans were measured and processed with standard Bruker software.

The Raman experiments were performed in a bath cryostat at a temperature of 1.2 K. To minimize Rayleigh scattering, glycerol (30 vol %) was added to the solution of the protein. The protein was irradiated with about 100 mW of light at 635.5 nm from a DCM dye laser (Coherent Model 599) which was pumped with an argon ion laser (Spectra-Physics Stabilite 2017). In detection, a double monochromator (Spex Model 1403, third slit opened) with a grating of 1800 lines/mm and a liquid nitrogen cooled CCD camera (EG&G Model 4000) were used. The camera was operated as a linear array (about 0.14 cm^{-1}/pixel), and the Raman spectra were composed of several measurements each corresponding to a range of about 60 cm^{-1}. The resolution was about 1 cm^{-1}. The spectra were calibrated with a neon lamp (Oriel spectral lamps).

Results and Discussion

Proton NMR spectra have been recorded for the reduced form of the proteins in H_2O and in D_2O and of the proteins reconstituted in D_2O. For *P. aeruginosa* azurin in D_2O and reconstituted in D_2O the NMR spectra are shown in parts a and b, respectively, of Figure 1. In the spectrum of native azurin in D_2O a number of bands are visible related to nonexchanged protons, among which the bands at 10.6 and 10.5 ppm that have been assigned to the amide protons of Asn47 and Phe114.[21] The exchange of the amide protons becomes almost complete for azurin when reconstituted in D_2O. A few bands remain, among those the ones assigned to the nonexchangeable C_ϵ and C_ζ protons of His35 and Phe15, respectively.[21]

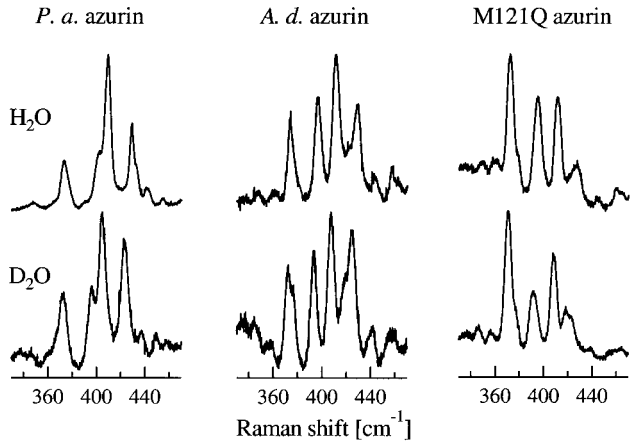

Figure 2. Resonance Raman spectra recorded at 1.2 K for *P. aeruginosa* azurin, *A. denitrificans* azurin, and the azurin mutant M121Q in H_2O (upper spectra) and reconstituted in D_2O (lower spectra).

The resonance Raman spectra between 350 and 460 cm^{-1} of the azurins in H_2O at 1.2 K are shown in the upper part of Figure 2. They agree with literature data[15,18,19,25] and reveal strong, partially overlapping bands and weaker ones at the low- and high-frequency sides. At about 800 cm^{-1} overtone and combination bands are observed (data not shown). The spectra in the lower part of Figure 2 concern the same proteins but reconstituted in D_2O. They are similar to those of the proteins in H_2O, which shows that the normal-mode structure underlying the bands around 400 cm^{-1} is largely conserved upon deuterium substitution. Most of the bands are significantly shifted to lower frequency, e.g., 1.7 to 5.9 cm^{-1} for *P. aeruginosa* azurin. Comparison of the Raman spectra of the azurins from *P. aeruginosa*, *A. denitrificans*, and the mutant shows that the shifts of the intense bands are correlated: a relatively small shift of about 1.8 cm^{-1} for the bands around 374 cm^{-1} and larger shifts for the bands around 400, 410, and 428 cm^{-1}. The shifts are summarized in Table 1.

The observed shifts are much larger than those for the native proteins in D_2O.[15,18] For example, the band at 428.9 cm^{-1} in the spectrum of *P. aeruginosa* azurin in H_2O is found 5.9 cm^{-1} lower in frequency after reconstitution of the apoprotein in D_2O, whereas the shift of this band is 1.2 cm^{-1} for the native protein in D_2O. The deuterium isotope shifts reported previously for *P. aeruginosa* and *A. denitrificans* native azurin in D_2O[15,18] are included in Table 1. These small shifts, about 1.5 cm^{-1} on average, have been observed by various groups and interpreted as arising from either the exchange of the protons of the copper-coordinated histidines[12,15] or the exchange of the backbone amide protons that form a hydrogen bond with the sulfur of the cysteine.[9,18,19] The proton NMR spectra (cf. Figure 1) reveal that these amide protons do not exchange when the native protein is incubated in D_2O. Hence, the small shifts of the bands in the Raman spectra of the native proteins in D_2O cannot result from deuteration of these amide protons and probably correspond to deuteration of the histidines.[12,15]

On the other hand, the changes in the Raman spectra observed in the present study for the apoproteins reconstituted in D_2O, with respect to both frequency shifts and intensity redistribution, may well derive from the exchange of the amide protons that form a hydrogen bond with the cysteine sulfur. First, the NMR spectra show that these protons do exchange when the apoproteins are incubated in D_2O. Second, of the other amide protons that only exchange when the apo form is incubated in D_2O, only those of His46, Cys112, and Thr113 are within 6 Å of the sulfur. These protons are farther away than the amide

TABLE 1: Resonance Raman Frequencies (cm^{-1}) for *P. aeruginosa* Azurin, *A. denitrificans* Azurin, and the Azurin Mutant M121Q, in H_2O and Reconstituted in D_2O[a]

					P. aeruginosa Azurin					
native	347.9		374.3		401.9	409.0		428.9	432.3	441.5	454.7
reconstituted	346.2		372.5		396.0	404.4		423.0		437.2	449.3
shift	−1.7		−1.8		−5.9	−4.6		−5.9		−4.3	−5.5
native shift[15]	−2.0		−0.9			−1.0		−1.2		−1.5	0
					A. denitrificans Azurin					
native	347.7	360.7	374.0		396.4	411.0	422.3	428.6		443.3	457.3
reconstituted	345.1	357.6	372.2	376.1	392.9	407.1	419.3	424.3		440.8	455.3
shift	−2.6	−3.1	−1.8		−3.5	−3.9		−4.3		−2.5	−2.0
native shift[18]	−2	−3	−0.4		0	−1.0		−0.4		0	−1.4
					A. denitrificans M121Q Azurin					
native	349.1	360.3	372.4		394.9	411.2		427.6		443.9	459.5
reconstituted	346.6	356.2	370.7		391.5	408.5	418.3	423.1		439.0	456.2
shift	−2.5	−4.1	−1.7		−3.4	−2.7		−4.5		−4.9	−3.3

[a] Also included are the corresponding deuterium isotope shifts. Literature values of the deuterium isotope shifts observed for the native azurins in D_2O have been reproduced from refs 15 and 18.

protons of Asn47 and Phe114, which are about 2.5 Å from the sulfur and coupling of the resonantly enhanced Cu−S(cys) vibration to the adjacent S(cys)−H vibration is most likely.

Relatively large isotope shifts upon reconstitution in D_2O have also been observed for amicyanin from *Paracoccus versutus* and the green nitrite reductase from *Alcaligenes faecalis* (data not shown). To the best of our knowledge, the literature of resonance Raman data on copper proteins reconstituted in D_2O is limited to stellacyanin from *Rhus vernicifera*.[12] Interestingly, the copper site of the azurin mutant M121Q mimics that of stellacyanin. The axial ligand is the same, a glutamine instead of the methionine commonly present in blue-copper proteins, and X-ray diffraction indicates a virtually identical structure of the metal site and the coordinated cysteine.[4,5] On the other hand, the resonance Raman spectrum of *A. denitrificans* M121Q is of the azurin type (cf. the resemblance of the spectra of the azurins in Figure 2) and is largely different from the resonance Raman spectrum of stellacyanin.[12] The isotope shifts of the native protein in D_2O and the protein reconstituted in D_2O were reported to be the same for stellacyanin.[12] The shifts are small, less than 2 cm^{-1}, although the cysteine sulfur probably is involved in hydrogen bonds to two amide protons (Asn47 and Val91 for stellacyanin[5]) as for the azurins. The similarity of the isotope shifts for stellacyanin in D_2O and reconstituted in D_2O might indicate that in this case the amide hydrogens involved in the hydrogen bonds to the cysteine sulfur become exchanged already for the native protein in D_2O. This is conceivable because the X-ray study[5] reveals that the copper site is much more solvent exposed for stellacyanin than for the azurin mutant M121Q. The exchange could be investigated by NMR spectroscopy. Alternatively, the similarity of the isotope shifts for stellacyanin in D_2O and reconstituted in D_2O might derive from the fact that the normal modes corresponding to the intense bands in the resonance Raman spectrum of stellacyanin are hardly sensitive to substitution of deuterium into the S(cys)−hydrogen bonds. The intensity in the resonance Raman spectrum of stellacyanin is concentrated in two bands in the low-frequency range at 347 and 385 cm^{-1}. In this wavenumber range the shifts are small also for the *P. aeruginosa* and *A. denitrificans* azurins. Large isotope effects for the latter proteins are particularly observed for the bands above 395 cm^{-1}, where the resonance Raman intensity is low for stellacyanin.

The coupling of the copper−sulfur and sulfur−hydrogen vibrations indicates that for a proper description of the electronic structure of the blue-copper site in azurin the amino acids Asn47 and Phe114, although not copper-coordinated, have to be included. Besides the present resonance Raman study, data from NMR, electron paramagnetic resonance (EPR), and density functional theory (DFT) calculations support the relevance of the sulfur−hydrogen bond with regard to the electronic structure. A significant contact shift was observed for the amide hydrogen of Asn47 in the proton NMR spectrum of oxidized azurin.[7] Pulsed EPR, both electron nuclear double resonance (ENDOR)[26] and electron spin-echo envelope modulation,[27,28] experiments revealed spin density on a backbone nitrogen, initially assigned as that of Cys112[26] or of His46.[27] Recent DFT calculations of the nitrogen hyperfine interactions in azurin indicate that the backbone nitrogen instead concerns the one of Asn47.[29] The latter interpretation is consistent with the orientation of the nitrogen quadrupole tensor as derived from ENDOR experiments.[26]

In summary, we have performed resonance Raman spectroscopy on the blue-copper proteins azurin of *P. aeruginosa* and of *A. denitrificans*, and the azurin mutant M121Q, in H_2O and reconstituted in D_2O. By reconstituting the apoproteins in D_2O, more protons exchange than when the native solution is incubated in D_2O. Among these protons are those that make hydrogen bonds to the sulfur of the copper-coordinated cysteine. Many deuterium isotope shifts are found to be significantly larger than those observed for the native proteins in D_2O, and the corresponding normal modes of the bands around 400 cm^{-1} most probably contain Cu−S−H bending character.

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