transformations are typical in cluster reactions with small molecules. Scission of the Mo–Mo bond in the transformation of **2** to **4** is interesting since Mo–Mo bonds are typically thought of as being stronger than Co–Co bonds.[16,17] Electrochemically, **2** exhibits a quasi-reversible oxidative wave at +0.65 V (peak separation, 180 mV) up to +1.0 V. On the other hand, **3** exhibits no oxidation wave up to +0.75 V.[4,18] Reactivity differences observed in this system and, perhaps, those of mixed-metal clusters in general might well be characterized by their redox chemistry.

As part of a study of the chemical properties of cationic cluster **4**, it was treated with hydride nucleophiles. Thus, the addition of 5 equiv of $NaBEt_3H$, $NaBEt_3D$, or $NaB(OMe)_3H$ at −78 °C to **4**, followed by warming to room temperature, regenerated **2** in yields ranging from 65 to 80%. If less than 5 equiv of hydride was used, **2** was only partially regenerated and unreacted **4** was observed by $^{31}P\{^1H\}$ NMR spectroscopy. Possible $NO^+$ reduction products, $NH_3$ and $H_2O$, could not be detected by mass spectrometry. Lithium triethylborodeuteride was used to determine if N–D or O–D products could be observed by $^2H$ NMR. This technique also proved unsuccessful. It is possible that ammonia was trapped in situ as an amine–borane complex. Not surprisingly, the mechanism of this very interesting transformation is unknown, since in general, hydride reagents react with transition-metal nitrosyl clusters with some complexity.[5,19-21]

In summary, dramatic differences in reactivity are observed between two closely related heteronuclear Mo–Co clusters, **2** and **3**. The addition of $NO^+$ to **2** involves the scission of the Mo–Mo bond concomitant with CO loss and formation of a novel $\mu_4$-$\eta^2$-NO-coordinated cluster. The 1,2-phenylene(bis-$\mu$-phosphido) ligand exhibits stability toward the highly oxidizing nitrosonium ion and appears to prevent fragmentation of the metal core. The novel $\mu_4$-$\eta^2$-NO coordination observed in **2** may serve as a useful model for NO activation on heterogeneous surfaces. Further investigations of these bis($\mu$-phosphido) heteronuclear clusters are in progress.

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**Supplementary Material Available:** Details of the crystal structure determination and tables of fractional coordinates and isotropic or equivalent isotropic thermal parameters, anisotropic thermal parameters for non-hydrogen atoms, bond lengths and angles, and torsional angles (non-hydrogens) for **4** (15 pages); table of observed and calculated structure factor amplitudes for **4** (36 pages). Ordering information is given on any current masthead page.

(16) Dissociation energies of homonuclear diatomic molecules $D_0°(M_2)$: Co–Co, 40 ± 6 kcal/mol; Mo–Mo, 97 ± 5 kcal/mol; Co–Mo, see ref 2, Chapter 3.

(17) For examples of site-specific M–M bond cleavage in heteronuclear clusters, see: (a) Shapley, J. R.; McAteer, C. H.; Churchill, M. R.; Biondi, L. V. *Organometallics* **1984**, *3*, 1595 (cleavage of a W–W bond was observed as the major product in the reaction of a mixed-metal $W_2Ir_2$ cluster with $C_2Ph_2$). (b) Roland, E.; Vahrenkamp, H. *Organometallics* **1983**, *2*, 183 (cleavage of the Co–Co bond in the reaction of a heteronuclear cluster, $Co_2Ru_2$, with $C_2Ph_2$).

(18) Cyclic voltammetry was determined with a Bioanalytical Systems BAS-100 (Bioanalytical Systems, West Lafayette, IN) electrochemical analyzer. Acetonitrile solutions of 0.1 M TBAP were used as supporting electrolyte. A Pt disk, Pt wire, and Ag wire were used as the working, auxiliary, and quasi-reference electrodes, respectively. The quasi-reference electrode was calibrated against the $Fe/Fe^+$ couple by addition of ferrocene. (Gritzner, G.; Kuta, J. *Pure Appl. Chem.* **1982**, *54*, 1527.) IR compensation was employed in all runs. All manipulations and CV measurements were made under dry argon. Both **2** and **3** exhibit very large irreversible oxidation waves at +1.18 and +0.92 V, respectively. These waves are ascribed to the oxidation of the *o*-phenylene ligand.

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## Site-Directed Mutagenesis Reveals That the Hydrophobic Patch of Azurin Mediates Electron Transfer

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Despite much recent effort, the experimental support for the various electron transfer (et) mechanisms for blue copper proteins like the azurins and plastocyanins has remained circumstantial.[1-3] Here, for the first time, direct evidence is reported, based on site-directed mutagenesis, which demonstrates the involvement of the so-called hydrophobic patch of *Pseudomonas aeruginosa* azurin in electron transfer. This patch is centered around the partly exposed Cu-ligand residue His117. We have substituted the conserved residue Met44, which is located next to His117, by a lysine. While the structural properties of this mutant azurin fall within the range encompassed by related blue copper proteins, there is a drastic effect on its function in et.

Two places on the protein surface of azurin have been suggested as candidates for exit and/or entry of an electron. The first one is a hydrophobic surface patch located around the Cu ligand His117, which presumably is involved in the reaction of azurin with nitrite reductase[4] and in the electron self-exchange (ese) reaction of azurin.[5,6] A second et patch, formed by an area around His35, might be involved in the reaction with cytochrome $c_{551}$.[4,7,8] His35 exhibits $\pi$-electron overlap with Cu ligand His46. Both His117 and the His35/His46 relay might serve as electronic links between the Cu and the redox partners of azurin. To establish the possible role of the hydrophobic patch in et, we have substituted Met44, a conserved residue[9] located next to His117, by a lysine by means of site-directed mutagenesis.

The presence of the Met44Lys (M44K) mutation shows up in an increase of the p*I* value (wild-type (wt) azurin, 5.6; M44K azurin, 6.6) as measured by isoelectric focusing. Moreover, the Met44 replacement is clearly seen in the $^1H$ NMR spectrum of the mutant in which the resonance assigned to the $\epsilon$-$CH_3$ group of Met44 has disappeared[10] (Figure 1).

The effect of the mutation on protein and active-site structure was further investigated by means of EPR, UV/vis, and NMR spectroscopy. The EPR spectra of M44K and wild-type (wt) azurin are shown in Figure 2. Compared to the wt, the $A_\parallel$ and $g_\parallel$ parameters of the mutant have changed from $58 \times 10^{-4}$ to $64 \times 10^{-4}$ $cm^{-1}$ and from 2.257 to 2.241, respectively. In the customary $D_{2d}$ model of type I Cu sites,[11] the observed changes in EPR parameters correspond with a change of about 1° in the ligand–Cu–ligand angles, which is within the range encompassed by various wt azurins.[3,12,13]

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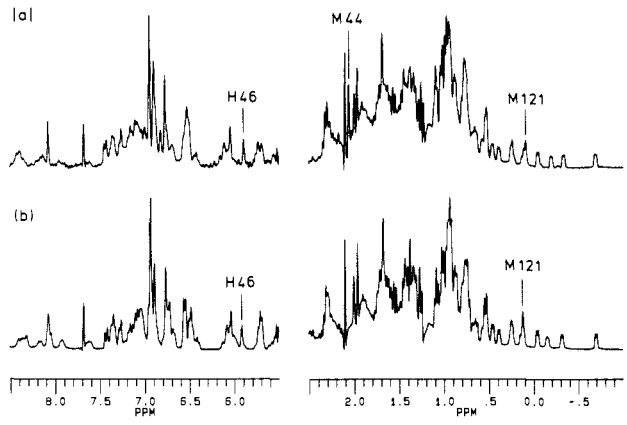


**Figure 1.** $^1$H NMR spectra of (a) 1 mM reduced wt azurin in 20 mM borate buffer pH* 9.0 at 314 K, and (b) 2 mM reduced M44K azurin in 20 mM phosphate buffer pH* 9.0 at 314 K, both in $D_2O$. (Asterisks indicate that the pH meter readings are not corrected for the deuterium isotope effect.) $^1$H NMR spectra were recorded on a Bruker WM-300 spectrometer. About 600 free induction decays/spectrum were accumulated in 8K memory, deconvoluted by Gaussian multiplication, and Fourier transformed. The horizontal scale was calibrated with tetramethylammonium nitrate. Only the aromatic and aliphatic parts of the spectra are shown. H46, M44, and M121 denote the resonance positions of the His46 C4H, Met44 $\epsilon$-$CH_3$, and Met121 $\epsilon$-$CH_3$ protons.[10] Notice that the M44 $\epsilon$-$CH_3$ resonance has disappeared in the spectrum of the M44K mutant. The mutant was constructed by in vitro oligo-directed site-specific mutagenesis using the selection method described by Kunkel.[15] The mutation was confirmed by ss- and ds-DNA sequencing.[16,17] Both wt and mutant azurins were isolated from *Escherichia coli* K-12 KMBL1164 cells (relevant genotype $\Delta$ *lac-pro*, F$^-$). The strain was transformed with pUC8 plasmids[18] in whose *Pst*I site the *Pst*I fragment containing the wt or mutant azurin gene[19,20] has been cloned. The EPR and $^1$H NMR spectra of heterologously expressed wt azurin were identical with those of the wt azurin isolated from *P. aeruginosa*.[10,13] The wt and mutant proteins were correctly processed as judged by their being localized in the periplasmic space and by their correct length as observed with SDS-PAGE (results not shown).

The red absorption band maximum of the M44K mutant occurs at the same wavelength as in the wt azurin (625 nm). The specific absorption coefficient at 625 nm was determined by a titration of the apoprotein with $CuSO_4$ and a redox titration of the reduced protein with $K_3[Fe(CN)_6]$[14] and amounts to 5200 $M^{-1}$ $cm^{-1}$. The redox potential has increased by 40–60 mV, consistent with the presence of an extra positive charge near the copper. The position and extinction of the red absorption band and the value of the redox potential are within the range of values normally encountered for the azurins.[3]

The $^1$H NMR spectra of reduced wt and reduced M44K azurin (Figure 1) exhibit a striking overall similarity, indicating again that the introduction of the mutation has caused at most only slight changes in the structure of the protein. For instance, resonances originating from ligands of the Cu (His46, Met121) and a number of high-field aliphatic resonances, which are very sensitive to structural distortions, have hardly shifted from their position in the wt azurin spectrum.[10] The similarity extends to other regions of the spectrum as well. The conclusion is that the M44K mutation affects the overall protein structure and the Cu-site structure in particular only slightly.

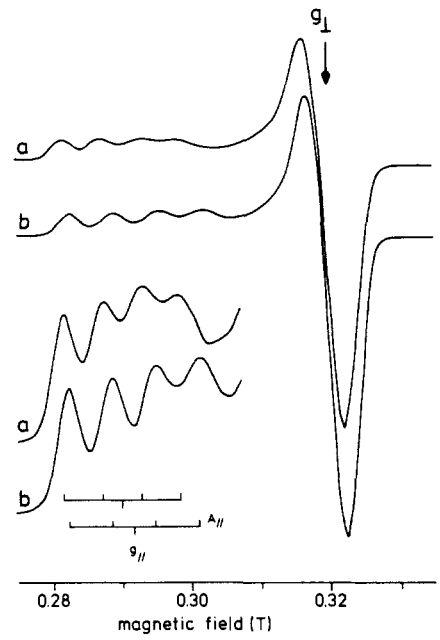


**Figure 2.** EPR spectra of 1.5 mM (a) wt and (b) M44K azurin at 77 K and pH 5.5 in 12 mM MES buffer containing 40% glycerol. The spectra were recorded with a Varian E-3 X-band EPR spectrometer at magnetic fields between 0.25 and 0.35 T with a modulation amplitude of 2 mT, at a frequency of 9.19 GHz. The magnetic field was calibrated with DPPH ($g$ = 2.0037).

The opposite obtains for the et kinetics. The ese rate constant $k_{ex}$ was determined at pH 5.0 and 9.0 at 309 K. This was done by introducing small amounts of oxidized M44K azurin into a solution of the reduced protein and measuring the broadening of a number of ligand proton peaks in the $^1$H NMR spectrum of the solution.[5,6] At pH 5.0, the line broadening was less than 1.0 rad/s at $[Az_{ox}]$ = 1.0 mM, leading to an upper limit of $k_{ex} < 1.0 \times 10^3$ $M^{-1}$ $s^{-1}$, while at pH 9.0, $k_{ex}$ was found to amount to about $1.0 \times 10^5$ $M^{-1}$ $s^{-1}$. Results of measurements at pH 9.5 appear compatible with a further increase of $k_{ex}$ with pH (at pH 9.5, 10–40% increase of $k_{ex}$ over its pH 9.0 value). On the other hand, for wt azurin at 309 K, $k_{ex}$ amounts to $1.3 \times 10^6$ $M^{-1}$ $s^{-1}$, independent of pH.[5,6] Thus, introduction of the single M44K mutation has nearly no effect on the 3D structure of azurin, but has a strong effect on its function. It is concluded that the hydrophobic patch is involved in the ese reaction, indeed. Preliminary results from experiments with another *P.a.* azurin mutant, in which His35 is substituted by glutamine, show that for this mutant neither $k_{ex}$ nor its pH dependence differs from that of wt azurin, indicating that the His35 patch is *not* involved in the ese reaction and thus constituting indirect support for the involvement of the hydrophobic patch in the ese reaction. It is tempting to presume that the observed pH effect on the ese rate of M44K azurin is correlated with the protonation of Lys44. This is the object of further research.

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**Registry No.** Met, 63-68-3; lysine, 56-87-1.

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# *Book Reviews**

**Recombinant DNA Methodology.** Edited by Ray Wu (Cornell University) et al. Academic: San Diego and New York. 1989. xxxi + 760 pp. $49.95. ISBN 0-12-765560-3.

This spiral-bound book consists of articles that previously appeared in Volumes 68, 100, 101, 153, 154, and 155 of *Methods in Enzymology*, selected because they contained theoretical discussion or experimental description that is still up-to-date and useful. The volumes from which they were selected were devoted to DNA research, and it is appropriate that the present book includes their Tables of Contents. An 18-page index is a welcome feature.

*Unsigned book reviews are by the Book Review Editor.