Table I. Normalized Cross-Peak Intensities in a Selective TOCSY-NOESY Experiment

	pathway:[a]	(^{n}NH-^{n}CH)>^{n}NH		(^{n}NH-^{n}CH)>$^{n+1}$NH		$^{n+1}$NH>^{n}CH	
^{n}peptide	sample:[b]	A	B	A	B	A	B
^{1}Gly				6.1[c]	3.8[c]	11.1[c]	12.9[c]
^{2}Cys		2.4	2.5	5.0	3.7	35.4	41.3
^{3}Phe		2.9	3.8	12.2	10.4	21.7	25.5
^{4}Val		8.1	6.8				
^{5}Pro						32.4	36.7
^{6}Cys		4.4	5.6	14.6	8.6	7.8	7.8
^{7}Gly		3.7	3.9				

[a] Magnetization transfer pathways are represented as follows: The leftmost NH corresponds to the selectively excited proton. TOCSY mixing occurs between the protons enclosed in parentheses, and the > sign represents NOE transfer in the direction indicated. The cross peak ($a > b$) appears at frequencies $\omega_1 = a$, $\omega_2 = b$. [b] Sample A: 64 mg of peptide was dissolved in 0.7 mL of d_6-DMSO. Sample B was obtained from A by partial exchange with D_2O. Degree of deuteration: 31%. Spectra were obtained in a Varian VXR-500 instrument with a 10-dB fixed attenuator at the output of the transmitter. The 270° Gaussian pulse was 2 ms and the 90° pulse width was 17 μs. TOCSY mixing: 30 ms (6.5 kHz). NOESY mixing: 300 ms. [c] Normalized to the $^{n+1}$NH intensity. Otherwise, normalized with respect to ^{n}NH intensity. Units are percent of the intensity of the reference peak. Intensities are the average of two experiments with the same samples. Reproducibility is better than 10%.

ferred back to the original NH in the same residue (intraresidue NOE) or to the symmetry-related NH proton (interresidue NOE). The latter interaction is suppressed because in the mixed dimers only one of the symmetry-related amide protons has been exchanged.

If x is the probability of one particular site to be deuterated, i.e., the degree of deuteration of the sample, the intensity of a purely interresidue cross peak in a semiselective TOCSY-NOESY experiment will be approximated by

$$I = I_0(1 - x)^2$$

where I_0 is the intensity of the cross peak in the fully protonated sample. The intensity of a purely intraresidue cross peak will be given simply by

$$I = I_0(1 - x)$$

which is the same dependency expected for the diagonal peaks. It follows that for purely intraresidue interactions the intensity ratio between cross and diagonal peaks will be independent of the degree of deuteration.[13]

The sequence has been tested by using the synthetic peptide dimer

H-Gly-Cys-Phe-Val-Pro-Cys-Gly-OH
| |
H-Gly-Cys-Phe-Val-Pro-Cys-Gly-OH

with two disulfide bridges that ensure a parallel orientation of the two chains.[14] The results are summarized in Table I. The cross peaks between CH_α in one sequence position and NH in a different one (colums 4 and 5) are attenuated by partial deuteration independently of whether the two residues belong to the same or a different chain. The last two columns in Table I (columns 6 and 7) show the normalized intensities of the cross peaks located on the opposite side of the diagonal. They arise from NOE transfer between NH protons which have been directly excited and neighbor CH_α protons as in an ordinary NOESY experiment, and as expected, they are not attenuated by deuteration. On the other hand, it can be seen that the normalized intensity of the cross peaks between CH_α protons, which have been prepared by TOCSY transfer from the selectively excited NH's and NH protons assigned to the same sequence position (columns 2 and 3) is independent of the degree of deuteration in all cases except for ^{4}Val. The decrease in intensity of the $CH_\alpha NH$ cross peak of this residue indicates that it contains a contribution from the interchain interaction between the two symmetry-related valine residues. This long-range interaction would have been overlooked in an ordinary NOESY experiment.

The selective TOCSY-NOESY experiment is a pseudo-3D NMR experiment. In a complete 3D experiment differentiation between intra- and interresidue $CH_\alpha NH$ NOEs would be obtained from the intensities of the back-transfer peaks as a function of the degree of deuteration.

Acknowledgment. We thank Dr. M. Ruiz-Gayo and Miss M. Royo for the peptide sample and Dr. M. Feliz and Dr. F. Albericio for helpful discussions. This work was partially supported by funds from the CICYT (PB90-257).

Registry No. GCFVPCG peptide dimer, 118972-74-0; GCFVPCG peptide dimer (deuterated), 133833-57-5.

(13) It should be stated that these intensity relationships are only an approximation to the true values as the presence of deuterons in the molecule is expected to affect the different cross-relaxation rates.

(14) Ruiz-Gayo, M.; Albericio, F.; Pons, M.; Royo, M.; Pedroso, E.; Giralt, E. *Tetrahedron Lett.* **1988**, *29*, 3845-3848.

Type I and II Copper Sites Obtained by External Addition of Ligands to a His117Gly Azurin Mutant

Tanneke den Blaauwen, Mart van de Kamp, and Gerard W. Canters*

Gorlaeus Laboratories, Leiden University
Leiden, The Netherlands

Received February 8, 1991

Low molecular weight copper complexes are often used as model compounds to study the coordination of Cu ions in proteins. Usually this approach meets with only partial success. Here a new strategy is reported by which a Cu site is made accessible in situ to different ligands by site-directed mutagenesis.

There has been much speculation recently on the mode of action of type I copper proteins,[1-7] in particular on the pathways followed by the electrons on their way to and from the active site inside the protein. A study of azurin mutants has recently provided strong support for the idea that in the electron self-exchange reaction as well as in the reaction of azurin with its presumed physiological partners (cytochrome c_{551} and nitrite reductase) His117 is the port of entry and exit for electrons.[5,6] The (unrefined) crystal structure of wild-type (WT) *Pseudomonas aeruginosa* azurin (reported at 2.7 Å[8]) and the refined structures of two His35 mutants (reported at 1.9-Å and 2.1-Å resolution[9]) show

* Address for correspondence: G. W. Canters, Gorlaeus Laboratories, PO Box 9502, 2300 RA Leiden, The Netherlands.

(1) Farver, O.; Blatt, Y.; Pecht, I. *Biochemistry* **1982**, *21*, 3556-3561.
(2) Farver, O.; Pecht, I. *Isr. J. Chem.* **1981**, *21*, 13-17.
(3) Silvestrini, M. C.; Brunori, M.; Wilson, M. T.; Darley-Usmar, V. M. *J. Inorg. Biochem.* **1981**, *14*, 327-338.
(4) Adman, E. T. in *Metalloproteins*; Harrison, P., Ed.; Verlag Chemie GmbH: Weinheim, 1985; Part 1, pp 1-41.
(5) van de Kamp, M.; Floris, R.; Hali, F. C.; Canters, G. W. *J. Am. Chem. Soc.* **1990**, *112*, 907-908.
(6) van de Kamp, M.; Silvestrini, M. C.; Brunori, M.; van Beeumen, J.; Hali, F. C.; Canters, G. W. *Eur. J. Biochem.* **1990**, *194*, 109-118.
(7) Groeneveld, C. M.; Canters, G. W. *J. Biol. Chem.* **1988**, *263*, 167-173.
(8) Adman, E. T.; Jensen, L. H. *Isr. J. Chem.* **1981**, *21*, 8-12.
(9) Nar, H.; Messerschmidt, A.; Huber, R.; van de Kamp, M.; Canters, G. W. *J. Mol. Biol.* **1991**, *218*, 427-447.

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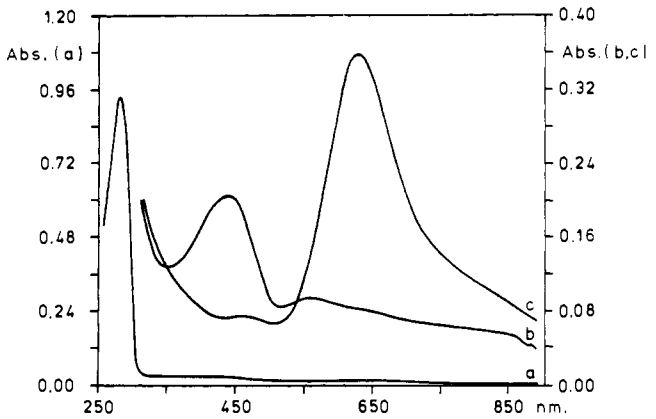


Figure 1. (a) Room temperature UV/vis spectrum (absorbance versus wavelength in nanometers) of a 0.1 mM His117Gly azurin sample in 16 mM MES buffer pH 6.0. (b) Same as spectrum a but after addition of a 10-fold excess of $Cu(NO_3)_2$. (c) Same as spectrum a but after addition of a 10-fold excess of $Cu(NO_3)_2$ and *N*-MeIm. The spectra were recorded on a Varian DMS spectrophotometer at 100 nm min^{-1}.

that His117 connects to the Cu, the Cu being located about 7 Å below the protein surface, and that the side chain of His117 pierces the protein surface at the location of the so-called hydrophobic patch.[9] The His side chain is not long or bulky enough, however, to protrude into the surrounding medium. Instead, when the protein is viewed from the outside, the protein surface at the hydrophobic patch exhibits a shallow depression in the middle of which the His117 side chain is visible. In the crystal structure this depression is filled by a water molecule anchored firmly to the protein by a hydrogen bridge to the N_ϵ of His117.[9,10] It has been suggested that this water molecule is also present in solution and could play a crucial role in facilitating electron transport to His117 and, thus, to the Cu.[9] To further study the details of the electron transport mechanism, we have replaced His117 by an amino acid with a small side chain (glycine), with the idea that this might create an aperture in the surface of the hydrophobic patch of the protein through which artificial Cu ligands might be attached directly to the copper. Here the first successful experiments along these lines are reported.

The His117Gly mutant was constructed through site-directed mutagenesis by using the selection method of Kunkel.[11] The mutant protein was expressed in *Escherichia coli* K12 JM101[12,13] and purified.[14] Preliminary ^1H NMR data of apo-His117Gly indicate that the overall structure of the mutant is similar to that of wild-type apoazurin (results not shown). Thus, introduction of the His117Gly mutation appears to have only small effects on the 3D structure of azurin. After addition of a 10-fold excess of $Cu(NO_3)_2$ to a 0.1 mM solution of the mutant at pH 6.0, a green color develops within a minute.[21] The optical spectrum exhibits a strong absorption around 420 nm (estimated extinction coefficient > 2.0 mM^{-1} cm^{-1}) instead of the characteristic 628-nm absorption of the WT azurin (Figure 1). The EPR spectrum shows a mixture of Cu sites, consisting presumably of a type II Cu center and Cu(II) bound nonspecifically.[15] The adventitiously bound Cu could be removed by ultrafiltration in the presence of 0.2 M NaCl, resulting in a homogeneous EPR spectrum that clearly corresponds to a type II center with an $A_\parallel$ of 139×10^{-4} cm^{-1} and a $g_\parallel$ of 2.283 (Figure 2). The perpendicular region exhibits a fine structure which may be due in part to rhombicity and in part to hyperfine splitting, the spacing of which (14×10^{-4} cm^{-1}) suggests a Cu coordinated by one or more N-donor ligands.[16]

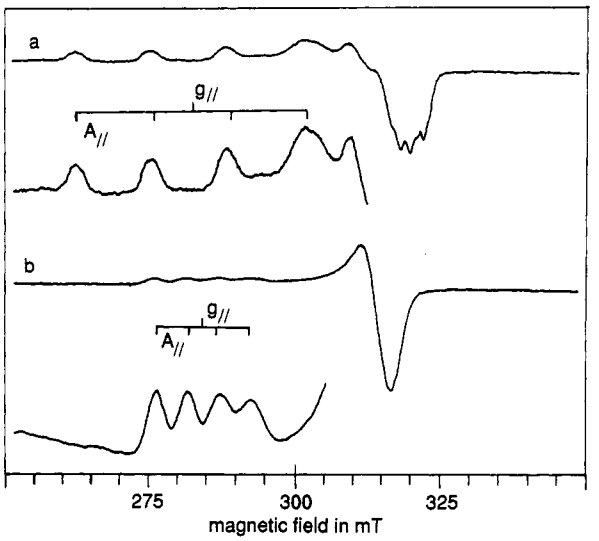


Figure 2. EPR spectra of a 2 mM His117Gly azurin sample at 77 K in 10 mM MES buffer pH 6.0 containing 40% glycerol, (a) incubated with copper and (b) incubated with copper and *N*-MeIm. The spectra were recorded with a Jeol JES-RE2X spectrometer operating at the X band and interfaced with an ES-PRIT330 data manipulation system. Parameters for recording EPR spectra were typically 125 G/min sweep rate, 6.3-G modulation amplitude, 8.950-GHz frequency, and 5-mW incident microwave power. The magnetic field was calibrated with DPPH (g = 2.0037).

After addition of $Cu(NO_3)_2$ and *N*-methylimidazole (*N*-MeIm) to the green protein solution, the characteristic blue color of the WT azurin immediately appears with the concomitant disappearance of the 420-nm band.[21] The corresponding red absorption band maximum occurs at almost the same position (630 nm) as in the spectrum of the WT (628 nm, Figure 1). An estimated lower limit of 4.7 mM^{-1} cm^{-1} for the extinction coefficient at 630 nm could be obtained from the spectrum by using the WT value of 9.8 mM^{-1} cm^{-1} for the extinction coefficient at 280 nm.[14] Also, the EPR spectrum of His117Gly containing copper and *N*-MeIm is hardly distinguishable from the WT EPR spectrum (Figure 2). Compared to the wild type, the $A_\parallel$, $g_\parallel$, and the $g\perp$ parameters of the mutant have changed from 58×10^{-4} cm^{-1} to 57×10^{-4} cm^{-1}, from 2.260 to 2.266, and from 2.058 to 2.055 at pH 6.0, respectively, values that are fully within the range encompassed by various wild-type azurins.[4,17,18]

The experiments reported here demonstrate that the type I Cu site in His117Gly azurin can be fully restored by the addition of Cu and *N*-MeIm. Apparently, the protein matrix does not collapse when His117 is removed and can still easily accept the imidazole ring when Cu has entered the metal site. The coordination of the Cu in the type II site reported here is intriguing. In WT azurin the Cu coordination has been described as trigonal bipyramidal with a strong equatorial N_2S coordination by His46, His117, and Cys112, and weaker axial coordination by the carbonyl oxygen of Gly45 and the S_δ of Met121.[8,10] The strong band at 420 nm in Figure 1b and the hyperfine structure observed in the $g_\perp$ region in Figure 2a are indications that Cys112[19,20] and His46,[16] respectively, are still involved in Cu coordination in the type II site studied here. The His117Gly mutation introduces conformational flexibility in the loop that carries three of the four Cu ligands in WT azurin, thus allowing for a possibly more pronounced role of O45 and S_δ121 in the coordination of the Cu in the type II site. It is conceivable that an additional group (e.g., H_2O or OH^-) filling

(10) Baker, E. N. *J. Mol. Biol.* **1988**, *203*, 1071–1095.
(11) Kunkel, T. A. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 488–492.
(12) Sanger, F.; Nicklen, S.; Coulson, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5463–5467.
(13) Vieira, J.; Messing, J. *Gene* **1982**, *19*, 259–268.
(14) Van de Kamp, M.; Hali, F. C.; Rosato, N.; Finazzi-Agro, A.; Canters, G. W. *Biochim. Biophys. Acta* **1990**, *1019*, 283–292.
(15) Ainscough, E. W.; Bingham, A. G.; Brodie, A. M.; Ellis, W. R.; Gray, H. B.; Loehr, T. M.; Plowman, J. E.; Norris, G. E.; Baker, E. N. *Biochemistry* **1987**, *26*, 71–82.
(16) Bednarek, J.; Schlick, S. *J. Am. Chem. Soc.* **1990**, *112*, 5019–5024.
(17) Peisach, J.; Blumberg, W. E. *Arch. Biochem. Biophys.* **1974**, *165*, 691–708.
(18) Groeneveld, C. M.; Aasa, R.; Reinhammar, B.; Canters, G. W. *J. Inorg. Biochem.* **1987**, *31*, 143–154.
(19) Kitajima, N.; Fujisawa, K.; Moro-oka, Y. *J. Am. Chem. Soc.* **1990**, *112*, 3210–3212.
(20) Downes, J. M.; Whelan, J.; Bosnich, B. *Inorg. Chem.* **1981**, *20*, 1081–1086.

the gap created by the mutation at position 117 has also entered the coordination sphere of the Cu.

Acknowledgment. We thank Jan Reedijk for valuable discussions during the course of this work. This research was supported by The Technology Foundation (STW), which is subsidized by the Department of Economic Affairs.

(21) In subsequent experiments we have found that excess Cu is not needed to produce absorption bands at 420 or 630 nm but that excess imidazole is needed to obtain maximal absorption at 630 nm. Preliminary experiments with other N-donor or O-donor ligands show that the H117G protein in the presence of Cu has a preference for N-donors and that the spectral features qualitatively resemble either the spectra represented here for the "Cu only" form or the "Cu + MeIm" form. Further experiments are in progress.

Simultaneous Determination of Counterion, Alcohol, and Water Concentrations at a Three Component Microemulsion Interface Using Product Distributions from a Dediazoniation Reaction

Arabinda Chaudhuri and Laurence S. Romsted*

Department of Chemistry, Wright and Rieman Laboratories
Rutgers, The State University of New Jersey
New Brunswick, New Jersey 08903

Received January 22, 1991

Solutions of association colloids such as micelles, vesicles, and microemulsions are composed of dynamic aggregates of surfactants and additives such as alcohols, salts, oils, and water.[1] All have highly anisotropic interfaces between their water and oil regions, and the interfacial concentrations of ions and molecules are usually several orders of magnitude greater than their overall concentrations in solution (Figure 1). Various methods are used to estimate the fraction of ions and molecules "bound" to association colloids, primarily from aggregate-induced shifts in a bulk solution property.[2] However, their local concentrations within the interfacial region can be calculated from their fraction bound only by making assumptions about their distributions within the aggregate and the volume of the interfacial region.[3]

We have developed a novel chemical method for estimating, simultaneously, the local interfacial concentrations of different nucleophiles, Y_m, in moles/liter of effective volume, at the surfaces of association colloids. Product distributions from dediazoniation of aggregate-bound, amphiphilic 16-ArN_2^+ (Scheme I), with its cationic head group oriented in the interfacial region like a surfactant molecule (Figure 1),[4] will be proportional to the local nucleophile concentrations in the interfacial region. To estimate Y_m, we assume that the selectivity of the dediazoniation reaction of 16-ArN_2^+ toward different nucleophiles, which cannot be measured independently, is the same as that of its short-chain analogue, 1-ArN_2^+, in aqueous solution.[4] Here we report the first simultaneous, experimental estimates of Br^-_m, H_2O_m, and $BuOH_m$ in cetyltrimethylammonium bromide, CTABr, microemulsions (Figure 2).

Arenediazonium salts are believed to decompose in aqueous acid in the dark via rate-determining formation of a very reactive

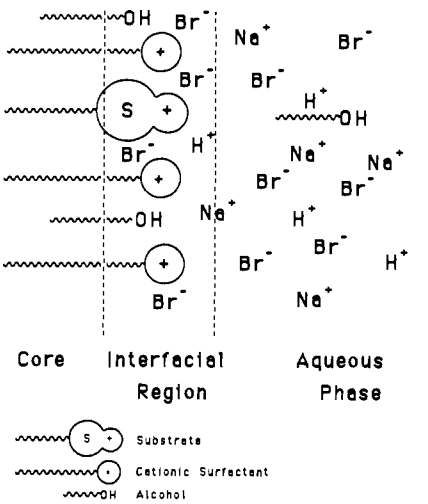


Figure 1. Cartoon of the interfacial region of a three-component microemulsion composed of a cationic surfactant, an alcohol, and an amphiphilic diazonium salt substrate, with its reactive group located in the interfacial region counterions, Br^-, and co-ions, Na^+ and H^+.

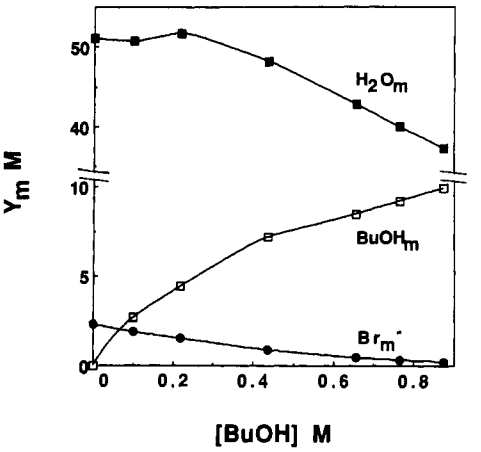
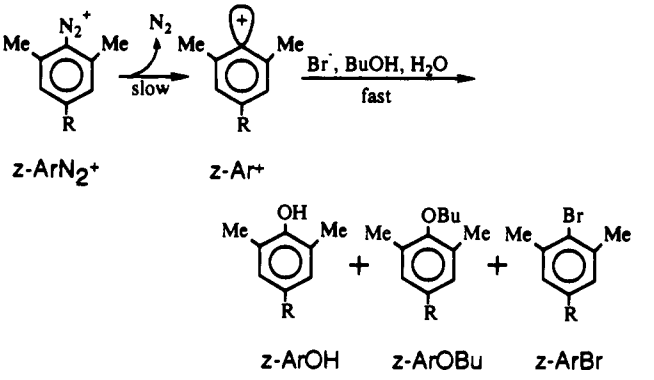


Figure 2. Effect of added BuOH on the local interfacial concentration, Y_m, of H_2O, Br^-, and BuOH; note ordinate scale change.

Scheme I[a]



[a] $z = 16$ when R = n-$C_{16}H_{33}$; $z = 1$ when R = CH_3.

aryl cation,[5,6] which traps available, weakly basic nucleophiles,[5] Scheme I. We assume that the observed selectivity of the dediazoniation reaction toward available nucleophiles[4,5] reflects the

(1) (a) Israelachvilli, J. N. *Intermolecular and Surfaces Forces*; Academic Press: New York, 1985. (b) Fendler, J. H. *Membrane Mimetic Chemistry*; Wiley-Interscience: New York, 1982. (c) Friberg, S. E.; Bothorel, P. *Microemulsion Structure and Dynamics*; CRC Press: Boca Raton, FL, 1987.

(2) (a) Zana, R., Ed. *Surfactants in Solution: New Methods of Investigation*; Marcel Dekker: New York, 1987. (b) Sepulveda, L.; Lissi, E.; Quina, F. *Adv. Colloid Interface Sci.* **1986**, *25*, 1.

(3) (a) Romsted, L. S. In *Micellization, Solubilization and Microemulsions*; Mittal, K. L., Ed.; Plenum Press: New York, 1977; Vol. 2, p 509. (b) Mukerjee, P. *J. Phys. Chem.* **1962**, *66*, 943.

(4) (a) Loughlin, J. A.; Romsted, L. S. *Colloids Surf.* **1990**, *48*, 123. (b) Loughlin, J. A. Ph.D. Thesis, Rutgers, The State University of New Jersey, 1990.

(5) (a) Swain, C. G.; Sheats, J. E.; Harbison, K. G. *J. Am. Chem. Soc.* **1975**, *97*, 783. (b) Hegarty, A. F. In *The Chemistry of the Functional Groups. The Chemistry of Diazonium and Diazo Groups, Part 2*; Patai, S., Ed.; Wiley: New York, 1978, pp 511-591. (c) Saunders, K. H.; Allen, R. L. M. *Aromatic Diazo Compounds*, 3rd ed.; Edward Arnold: Baltimore, 1985.

(6) (a) Scaiano, J. C.; Kim-Thuan, N. *J. Photochem.* **1983**, *23*, 269. (b) Cacace, F. *Science* **1990**, *250*, 392.

0002-7863/91/1513-5052$02.50/0