O16

The Redox Properties of Azurin from *Pseudomonas Aeruginosa* as Studied by High Frequency Proton NMR

E. T. ADMAN

Department of Biological Structure, University of Washington, Seattle, U.S.A.

G. W. CANTERS

Gorlaeus Laboratories, State University Leiden, The Netherlands

H. A. O. HILL and N. A, KITCHEN

Inorganic Chemistry Laboratory, South Parks Road, Oxford, U.K.

Since their 3-dimensional structures have become available [1–3] the blue copper proteins plastocyanin (Pc) and azurin (Az) have gained increased attention from spectroscopists [4]. In both proteins the Cu atom appears to be coordinated by 2 histidines, a cysteine and a methionine, and also in other respects the two structures exhibit a remarkable similarity. Yet in their electron transfer properties and their conformational behaviour as a function of pH Az and Pc show quite notable differences.

In Pc the Cu-coordinating His-87 becomes protonated at low pH (pH < 5.4) [1] and moves away from the metal, thus producing a 3-coordinated copper(I) centre. This leads to stabilization of the Cu(I)-state and loss of redox-activity. Moreover, Pc exhibits a large variation in the rate of electron transfer with other proteins (including itself) [5–7], which has been considered as indicative of specific protein-protein interactions. The existence of charged patches on the protein surface is in accordance with the purported electrostatic nature of these interactions [5].

In the case of Az, on the other hand, it was only known, that the protein undergoes a switch from a redox inactive to a redox active state when the pH drops below about pH = 7 [8]; we therefore started a proton NMR study of the redox properties of Az, the results of which are summarized here.

By studying the effect of slight oxidation on the NMR spectrum of Az from *Pseudomonas aeruginosa* the proton signals of the ligand residues His-46, His-117 and Met-121 were identified and subsequently their pH behaviour was studied [9]. As an example the case of Met-121 is shown here. The signal (labeled M_6) of the ϵ-methyl group of this residue at low pH appears at –0.05 ppm from TSS. The unusual upfield shifted resonance position is the result of the combined ring current effects of Phe-15 and His-46. With increasing pH the signal slowly loses intensity and at pH > 8 has disappeared. This was ascribed

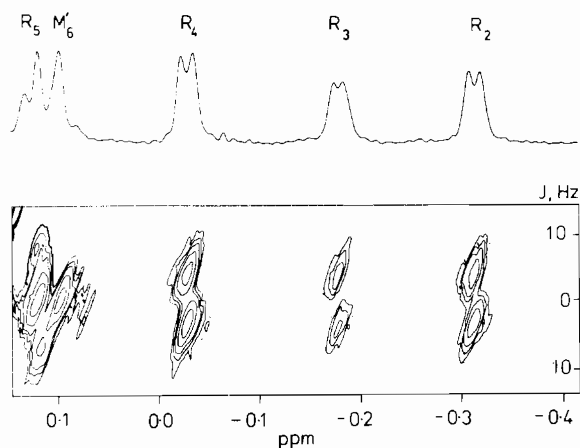

Fig. 1. High field portion of the 500 MHz NMR spectrum of a 2 m*M* solution of Az in D_2O at 42 °C and pH* = 8.06. Concentrations of phosphate buffer and NaCl: 20 m*M* and 100 m*M*, respectively. *Top*: normal NMR spectrum obtained after Gaussian multiplication of 800 FID's stored in 16 K memory and extended with zero filling to 32 K before Fourier transformation. *Bottom*: contour plots of 2-D J-resolved spectrum. The FID data matrix consisted of 32 × 8192 data points and was transformed by Gaussian multiplication along the t_2-axis and sine bell multiplication along the t_1-axis. Frequency resolution: 0.814 × 0.814 Hz/pt; number of FID s in each row: 512. The multiplets R_2, R_3, R_4 and R_5 have previously been assigned to the methyl groups of Val-22 $C_{\gamma 2}$, Val-31 $C_{\gamma 1}$, Leu-102 $C_{\delta 1}$ and Ile-7 $C_{\delta 1}$ [12], in that order.

tentatively to a weakening of the Cu–S (Met-121) bond and increased motional freedom of the Met-121 methyl group leading to a broadening of the NMR-signal [9]. Our recent experiments show that when peak M_6 disappears a new singlet appears (labeled M_6') at 0.11 ppm that overlaps with the $C_{\delta 1}$-methyl triplet signal of Ile-7 (labeled R_5). The singlet nature of M_6' is demonstrated in the 2-dimensional J-resolved spectrum reproduced in the figure. It is clear therefore, that a conformational change of the protein occurs when the pH is varied, which affects the position of the ϵ-methyl group of Met-121 with respect to Phe-12 and/or His-46. Saturation transfer experiments, to be detailed elsewhere, prove that the signals M_6 and M_6' are in slow exchange. Further study of the other ligand signals provides additional evidence that Az may exist in two conformations which interconvert on a time scale of 10–100 ms and that the residues His-35, His-46 and Met-121 are involved in the interconversion. On combining the crystallographic and the NMR data, it transpires that the function of His-35 probably is that of a pH-dependent relay between the Cu coordination shell and the protein surface.

In the course of the oxidation experiments it became apparent that the broadening of the NMR

signals induced by the paramagnetism of the Cu(II) species would yield information about the rate of electron self exchange of Az. A thorough analysis of the experimental data showed that, for a few NMR signals, the broadening could be analyzed according to the 'strong pulse limit'. In this limit the broadening is completely determined by the lifetime of the protein in the diamagnetic state and this leads directly to a value of the rate of electron self exchange, k. Although not very accurate (estimated accuracy ±50%) the value of 2×10^6 M^{-1} s^{-1} found at 50 °C for k in this way [10] clearly is in agreement with the self exchange rate inferred by Wherland and Pecht from the rate of electron transfer between Az and a variety of other redox proteins [6], though not with the data calculated by Gray and coworkers on the basis of a Marcus treatment of the heterogeneous electron transfer between Az and a series of inorganic transition metal compounds [11]. The high rate of self exchange of Az as well as the relatively fast electron exchange between Az and other redox proteins seems to indicate that non-specific hydrophobic interactions govern the reaction of Az with its reaction partners. This is consistent with the findings from Cr titration experiments [12] and with conclusions from the crystallographic work [2], that there are no pronounced charged patches on the Az surface.

1 P. M. Colman, H. C. Freeman, J. M. Guss, M. Murata, V. A. Norris, J. A. M. Ramshaw and M. P. Venkatappa, *Nature, 272,* 319 (1978).
2 E. T. Adman, R. E. Stenkamp, L. C. Sieker and L. H. Jensen, *J. Mol. Biol., 123,* 35 (1978).
3 E. T. Adman and L. H. Jensen, *Isr. J. Chem., 21,* 8 (1981).
4 T. G. Spiro, Ed., 'Copper Proteins', Wiley, Chichester (1981).
5 S. Niwa, H. Ishikawa, S. Nikai and T. Takabe, *J. Biochem., 88,* 1177 (1980).
6 S. Wherland and I. Pecht, *Biochemistry, 17,* 2582 (1976).
7 J. K. Beattie, D. J. Fensom, H. C. Freeman, E.Woodcock, H. A. O. Hill and A. M. Stokes, *Biochim. Biophys. Acta, 405,* 109 (1975).
8 M. C. Silvestrini, M. Brunori, M. T. Wilson and V. M. Darley-Usmar, *J. Inorgan. Biochem., 14,* 327 (1981).
9 E. T. Adman, G. W. Canters, H. A. O. Hill and N. A. Kitchen, *FEBS Letters, 143,* 287 (1982).
10 G. W. Canters, H. A. O. Hill, N. A. Kitchen and E. T. Adman, to be submitted.
11 D. Cummins and H. B. Gray, *J. Am. Chem. Soc., 99,* 5158 (1977).
12 E. T. Adman, G. W. Canters, H. A. O. Hill and N. A. Kitchen, in preparation.

O17

Specific Labeling of Iron–Sulfur Cluster Subsites

I. MOURA, J. J. G. MOURA and A. V. XAVIER

Centro de Química Estrutural, Complexo I, IST, Av. Rovisco Pais, 1000 Lisbon, Portugal

Desulfovibrio gigas ferredoxin is isolated in different oligomeric forms [1]. These ferredoxins, termed FdI and FdII, are built from one type of monomeric unit of which the sequence of 57 amino acids is known [2, 3]. FdII is a tetramer of molecular weight 24,000. Mössbauer and EPR studies [4] have shown that each monomer of FdII contains one [3Fe-xS] center. The other oligomeric form, the trimeric FdI [5] was shown to contain as a majority species a [4Fe–4S] cluster. However the FdI trimer can accomodate a [3Fe–xS] cluster as a minority species of variable proportions. Evidence was accumulated that both clusters can be accomodated by the polypeptide chain of this ferredoxin:

(a) Controlled reconstitution experiments of the active center of *D. gigas* FdII (containing only [3Fe–xS] cores) were performed in such a way that a reconstituted protein, Fd_R, loaded only with [4Fe–4S] cores was obtained [6].

(b) Mössbauer and EPR spectroscopies have been used to extensively study the process of cluster interconversion in *D. gigas* FdII. The [3Fe–xS] centres could be converted into [4Fe–4S] clusters after incubation with Fe^{2+} in the presence of dithiothreitol [6].

Based on these observations incubations of FdII were performed using 95% enriched ^{57}Fe in the presence of sulfide and dithiothreitol. Conversion from a [3Fe–xS] into a structure with a [4Fe–4S] core occurred; the latter seems to be structurally identical to the cluster of Fd_R. The ^{57}Fe Mössbauer

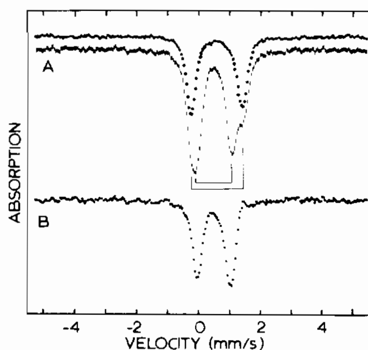

Fig. 1. (A) Mössbauer spectra of a reduced sample of FdII incubated with ^{57}Fe, sulfide and dithiothreitol (full circles) and of reduced reconstituted Fd (hatch marks). The spectra were recorded at 90 K in zero magnetic field. (B) Difference spectrum obtained by subtracting the spectrum of the incubated sample (full circles) from that of reduced Fd_R.