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Structure and reactivity of type-I copper sites

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Summary. Biological electron transfer is not well understood. The question is addressed in this contribution with reference to the so-called blue copper proteins, each of which has a single copper atom at its active centre. The redox activity (as probed by the electron self exchange reaction) of the Cu centre seems not to be affected. The electron self exchange reaction is known to proceed through His-117, and the hydrophobic patch is most important in the formation of the azurin/azurin encounter complex. Ph effects have not been observed on the three-dimensional structure of *A. denitrificans* azurin, which may indicate that if present at all these have no direct physiological implications. Mutants are in process of construction.

Key words: Electron transfer – Electron flow – Substrate conversion – Blue copper proteins

Introduction

In its mechanistic details biological electron transfer (ET) is not well understood. Electron flow *in vivo* is maintained primarily through the action of proteins, some of which generate or absorb electrons in the process of substrate conversion, some of which solely serve the purpose of the transport of electronic charge. The active centre of redox enzymes and electron transport proteins usually consists of a prosthetic group and/or one or more metal ions. Supposedly, during electron transport, the electrons are hopping from centre to centre. The question is: what are the crucial parameters that govern this flow of electronic charge?

In this contribution the question will be addressed by considering the so-called blue copper proteins, which have a single copper atom in their active centre. In particular, attention is paid to the following aspects.

a) Coordination chemists are familiar with the difference in coordination chemistry between the two more common redox states of Cu, Cu(I) and Cu(II). It has been stated (Vallee and Williams 1968) that the so-called type-I Cu centres have been 'designed' such that their coordination is a compromise between the coordination geometries of the Cu(I) and the Cu(II) state (tetrahedral vs square planar). In this way the kinetic barrier separating the reactants before and after ET, is minimized. The intriguing point is: to what extent and in what manner does the protein matrix (fine-)tune the properties of the Cu site (redox potential, ET rate, pH sensitivity)?

b) Since the active site is buried inside the protein, the electron has to traverse (part of) the protein matrix to reach or leave the active centre. One is interested to know whether there are preferred pathways for the electron inside the protein or whether electron transport occurs along a more or less random path.

c) It is usually assumed that for successful ET between physiological partners the proteins involved have to dock in a special way. Important questions germane to this aspect of biological ET concern the structure of the docking complex and the extent to which the efficiency of the ET is affected by the accuracy of the docking.

The presence of a single 3d metal ion in their active site makes the blue copper proteins particularly suited for investigations by spectroscopic techniques, like EPR, EXAFS and optical spectroscopy. In addition NMR has proven its value for the investigation not only of the details of the active-site structure, but also of the structure of the protein as a whole. Apart from the study of structural details, these techniques lend themselves naturally for mechanistic investigations. A recent and powerful addition to the armamentarium of the biochemically interested spectroscopist is site-directed mutagenesis by which protein residues can be varied almost at will. As a complement to this technique molecular dynamics calculations are used with increased frequency to predict or analyse the effects of mutations.

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The three points mentioned above will be elaborated upon in a discussion of the results of recent studies on three blue copper proteins, to wit amicyanin from *Thiobacillus versutus*, and the azurins from *Pseudomonas aeruginosa* and *Alcaligenes denitrificans*. In particular, the effect of pH on structure and activity is considered. Although their physiological meaning is not clear, these pH effects illustrate in a clear fashion the importance of the various points mentioned above.

Blue copper proteins

Blue copper proteins function as charge carriers in the electron transport chains of obligate and facultative anaerobic bacteria. They fulfil a similar role as the cytochromes, with which they often operate in series or in tandem. The single Cu atom in the so-called type-I Cu site appears to be coordinated (with very few exceptions) by two ring nitrogen atoms from two histidine side-chains, and two sulphur atoms from a cysteine and a methionine side-chain (Guss et al. 1986; Adman 1986; Baker 1988). Various sub-classes of the blue copper proteins can be distinguished on the basis of the length of the polypeptide chain, the primary sequence of the loop containing three of the four Cu ligands, and the type of redox chain in which the protein functions. Also the position of the single short α-helix (when present) in the secondary structure can help to distinguish the various sub-classes. In some cases a single domain of a multi-domain redox enzyme exhibits resemblance with a blue copper protein. In cytochrome *c* oxidase, for instance, the 'blue copper domain' might fulfil a role in the electron-transport-coupled translocation of protons over the mitochondrial or bacterial inner membrane (Palmer 1987; Mitchell 1987). In this respect the pH effect on structure and activity of blue copper proteins may have added interest.

Amicyanin from *T. versutus*

The first report on the isolation and identification of an amicyanin appeared in 1981 (Tobari and Harada 1981). In the meantime amicyanins have been isolated from various methylotrophs (Anthony 1989), in which they appear as redox partners of methylamine dehydrogenases. Amicyanins are about 100 amino acid residues long and contain a copper ligand loop Cys-Xaa-Xaa-His-Xaa-Xaa-Met, which seems particularly tight in comparison with, for instance, the longer loops in the plastocyanins, pseudo-azurins and azurins.

The Cu in amicyanin from *T. versutus*, which will be dealt with here, is coordinated by His-54, Cys-93, His-96 and Met-99. His-54 and His-96 are the only two histidines in the amino-acid sequence. The peculiar thing is that one of them, although being a ligand, titrates with pH ($pK_a = 7.3$ in D_2O at 297 K), as observed in the ^{1}H-NMR spectrum of the reduced protein (see Fig. 1). Preliminary results from two-dimensional NMR studies

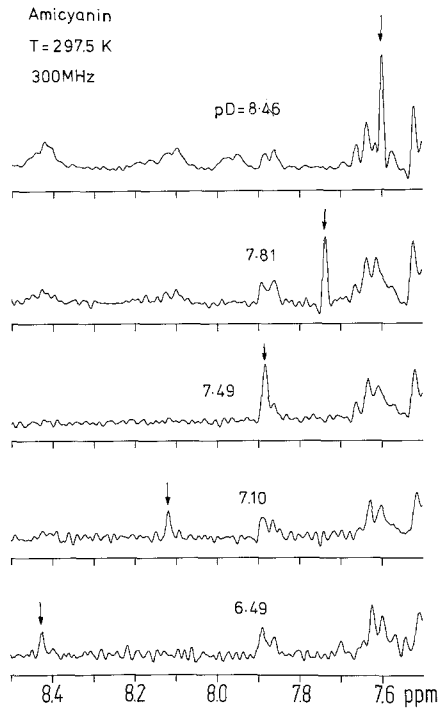

Fig. 1. Downfield region of the 300-MHz ^{1}H-NMR spectrum of a 1.5 mM solution of reduced amicyanin in D_2O, 20 mM potassium phosphate, 297.5 K, at various pD. The arrow denotes the ^{1}H-NMR signal of the C2 proton of the titrating ligand histidine. Resolution was enhanced by Gaussian multiplication; actual line widths were measured in untreated spectra

indicate that it probably concerns His-96. Apparently one of the Cu-His bonds is labile. Indirect evidence from ultraviolet/visible, ^{1}H-NMR and EXAFS spectroscopy on oxidized native amicyanin and Hg-amicyanin (Cu replaced by Hg) shows that the metal-histidine bond is not pH-labile, at least down to pH 4.5, in the oxidized [Cu(II)] amicyanin.

The pH behaviour of the C2 proton of the labile histidine in reduced amicyanin has been studied in detail as a function of temperature and spectrometer frequency (300 and 600 MHz). A plot of the chemical shift of this proton vs pD (all NMR measurements were performed in D_2O) appears normal, but the line width as a function of the fraction of the deuteronated species is not (see Fig. 2). At high pD the line width has a normal value of about 10 rad/s (half-width at half-height). With decreasing pD, the line width increases slightly at first but beyond $f_{HisD^+} = 0.5$ (f_{HisD^+} is the fraction of the deuteronated species) there is a considerable and continuous increase in line width. Extensive computer simulations based on the formalism of Patterson and Ettinger (1960) have shown that the line width data can be satisfactorily fitted (see Fig. 2) on the basis of an exchange between three sites A, B and C according to the scheme $A \rightleftharpoons B \rightleftharpoons C$. Here A represents the dedeuteronated species, and B and C represent two forms of the deuteronated species. The A/B equilibrium involves the exchange of a deuteron between the solution and the ligand histidine. The exchange occurs in the so-called fast exchange limit, and it is this exchange which

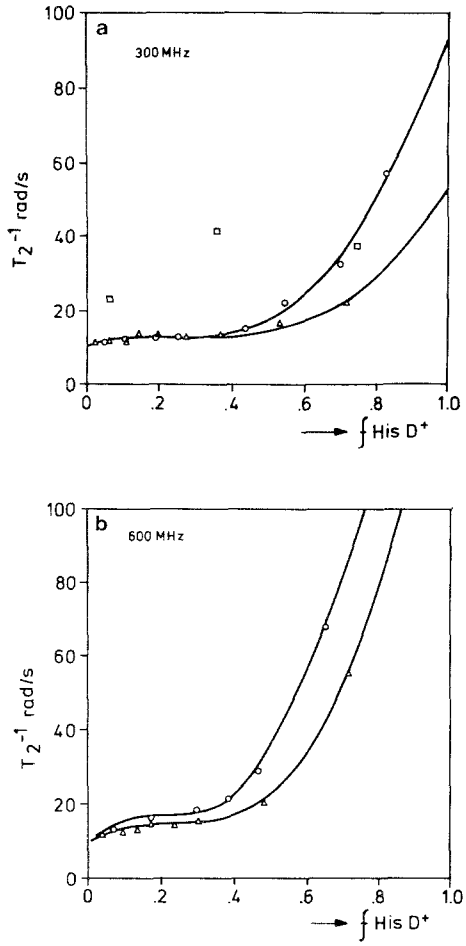

Fig. 2. Half-width at half-height of the C2 ^{1}H-NMR signal (in rad/s) as a function of the fraction of deuteronated amicyanin (f_{HisD^+}). Experiments were performed in 20 mM phosphate at (a) 300 MHz and (b) 600 MHz. (○) $T=317$ K; (▲) $T=297.5$ K; (□) measured in the absence of phosphate (unbuffered solution). The solid lines are simulations based on the three-site exchange model discussed in the text and on the theory of Patterson and Ettinger (1960)

is responsible for the (small) broadening effects seen at low values of f_{HisD^+}. The B/C equilibrium, on the other hand, involves an intramolecular transition and is shifted to the side of the B species ([C]/[B]≈0.2). The transition occurs in the slow exchange regime and causes the large increase in line width as the fraction of deuteronated species approaches the value of 1. The first-order rate constant for the B→C transition (250 s^{-1}, 297 K) is of the right order of magnitude to be compatible with an intramolecular reorientation of the ligand histidine. A somewhat speculative model for the A/B/C transitions, partly based on the analogy with plastocyanin (Guss et al. 1986), is presented in Fig. 3. The formulation of the A→B transition as a deuteron association step is not compatible with the observed pseudo-first-order transition rate of 10^5 s^{-1} at pD 7, since this would lead to a second-order reaction rate of 10^{12} M^{-1} s^{-1}. This is faster than the diffusion-controlled limit. However, experiments in which the (phosphate) buffer concentration was varied showed that the A/B transition is phosphate-catalysed (see Fig. 2). It appears that the A/B equilibrium can be reformulated in a manner consistent with the experimental findings by considering deuteron exchange between the protein and the $D_2PO_4^-$ and DPO_4^{2-} species present in the solution.

The foregoing results demonstrate that one is dealing here with a blue copper protein in which the protein matrix does not provide for a 'pH-proof' surrounding of the active site: at low pH there is a clear difference between the Cu(I) and the Cu(II) coordination geometry. What effect does this have on the redox activity of the amicyanin? To this end the electron self exchange (ESE) rate was determined by NMR methods at high and low pH and at different temperatures and values of the ionic strength. Summarized, the experimental findings show that at pD 8.6 the ESE rate amounts to 1.3×10^5 M^{-1} s^{-1} ($I=0.05$ M, 298 K), while for the acidic form of the amicyanin the rate was found to decrease to $<10^3$ M^{-1} s^{-1}. The results were interpreted as showing that fast ET is possible between reduced and oxidized amicyanin at pH values for which the Cu ion is four-coordinated in both species. At low pH, on the other hand, the ET is inhibited: the dissociation of a proton is required prior to the ET between two amicyanin molecules. Apparently the encounter complex between two amicyanins does not have the properties which are conducive to this kind of exchange during the life time of the encounter complex. This does not

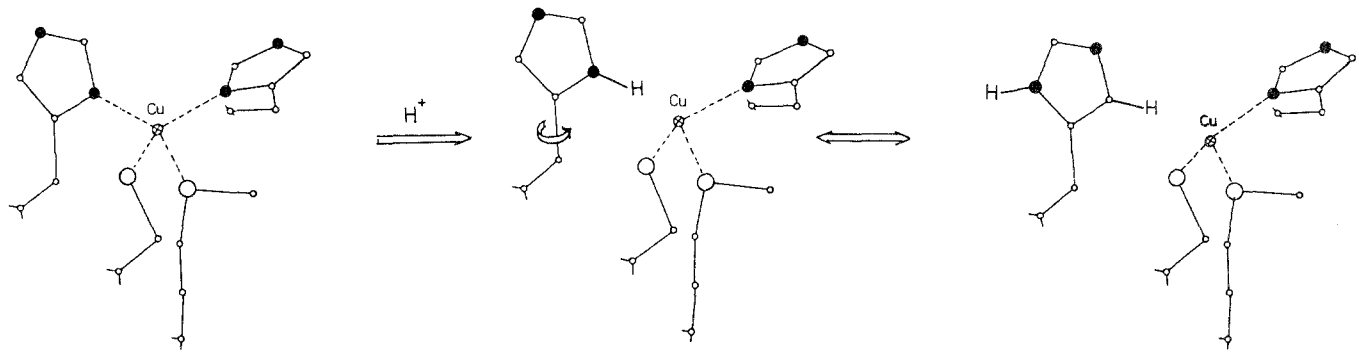

Fig. 3. Model illustrating the possible protonation and subsequent reorientation of the titrating ligand histidine in amicyanin as a function of pH. The three conformations correspond with the species A, B and C in the text

necessarily mean that the ET between amicyanin and its physiological partner is blocked at low pH, since the docking complex may be adapted to the simultaneous transfer of an electron and a proton, or to the transfer of an electron and the simultaneous uptake of a hydroxyl ion.

Azurin from *P. aeruginosa*

Azurins have been found in the respiratory chains of various denitrifying bacteria. The azurins sequenced so far have amino acid chains of 128 and 129 residues length (Ryden and Lundgren 1976). Cu ligands are the residues His-46, Cys-112, His-117 and Met-121. The kinetics of the redox reaction of *P. aeruginosa* azurin and cytochrome c_{551} has been found to depend on pH (Antonini et al. 1970) [for reviews see also Adman (1986) and Farmer and Pecht (1981)]. This has puzzled many research groups over the years (Rosen et al. 1981; Mitra and Bersohn 1982; Corin et al. 1983). The simplest scheme that has been proposed to explain the kinetic observations is one in which reduced (but not oxidized) azurin may exist in two conformations, only one of which would be redox-active (Silvestrini et al. 1981). The interconversion of the two conformations would involve a pH-dependent equilibrium.

Although the similarity with the amicyanin case seems striking at first sight, the likeness is only superficial. From extensive research by various groups (Antonini et al. 1970; Rosen et al. 1981; Silvestrini et al. 1981; Adman et al. 1982; Mitra and Bersohn 1982; Corin et al. 1983; Groeneveld and Canters 1988) it has gradually transpired that there is indeed a conformational change of the azurin induced by a change in pH (see Fig. 4). This conformational transition is triggered by a change in the state of protonation of His-35, which is not a ligand of the Cu but which occurs in the second coordination shell of the metal. His-35 has π-electron overlap with the Cu ligand His-46 and when it becomes protonated it rearranges its hydrogen bonding pattern, as a result of which the residues in the first coordination shell of the Cu are slightly reshuffled. At first it was thought that this reshuffling would switch off the redox activity of the Cu site at high pH and thus would explain the kinetic observations for the azurin/cytochrome c_{551} electron exchange. However, this explanation appeared untenable after it was found that the ESE rate for azurin was independent of pH (Groeneveld and Canters 1988). The Cu site is not 'switched off', apparently, at high pH; in fact the ESE rate appears to be one of the highest ever observed for blue copper proteins ($9.6 \times 10^5\ M^{-1}\ s^{-1}$ at 298 K, pH 4.5; Groeneveld and Canters 1988). Circumstantial evidence was reported for the involvement of the Cu ligand His-117 in the ESE reaction. This residue slightly protrudes through the so-called hydrophobic surface patch of the protein and might serve to communicate between the copper and the outside world. The findings for the cytochrome c_{551}/azurin kinetics, on the other hand, were tentatively explained by assuming that the surface

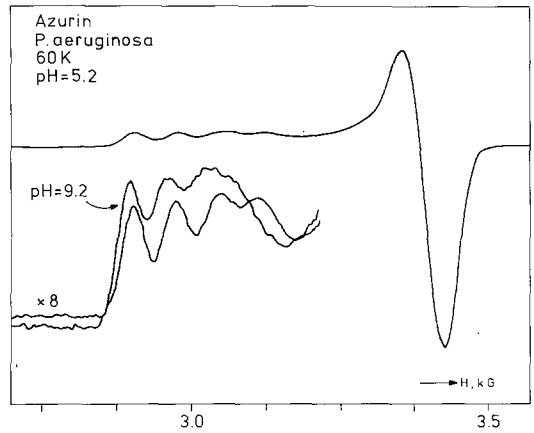

Fig. 4. EPR spectrum of oxidized azurin (concentration about 1 mM) in water at 60 K and pH 5.2 (20 mM Mes buffer). The inset shows the 8× vertically enhanced $g_{//}$ region of the spectrum; it also shows the same region of the pH-9.2 spectrum (20 mM borate buffer) measured under otherwise identical conditions. When applying the D_{2d} model of Brill (1977), the odd appearance of the $g_{//}$ region (uneven spacing and width hyperfine components) appears to be due to a spread of the ligand-Cu-ligand angles, β, of about 1–1.5°. Further, the average value of β is found to differ by about 1° for the high-pH and low-pH forms (see also Groeneveld et al. 1987)

patch around His-35 may be involved in the azurin/cytochrome c_{551} electron exchange. In this case the His-35/His-46 relay would function as a pH-dependent link between the Cu and the cytochrome.

This hypothesis lends itself for testing through site-directed mutagenesis. By studying the effect of mutations on the kinetics of the ESE reaction and the azurin/cytochrome electron exchange, an idea might be gained about how the electrons enter and leave the azurin. The results of the first experiments along these lines are summarised here.

The azurin gene was located on a *Pst*I fragment derived from the bacterial chromosome and subsequently cloned and sequenced (Canters 1987a; 1987b). The genome organization of the *Pst*I fragment is presented in Fig. 5. The azurin gene is preceded by its own promoter(s) and followed by a bi-directional terminator. It is coded in the form of a pre-protein in which the azurin peptide chain is preceded by a 19-amino-acid signal sequence. The signal peptide is needed for the translocation of the azurin over the cytoplasmic membrane into the periplasmic space. Parts of two possible open reading frames, which are accompanied by the expected regulatory elements and whose transcription direction is opposite that of the azurin gene, are located on either side of the blue copper gene. It is not known yet whether they are part of genes coding for proteins which belong to the same respiratory chain as the azurin.

Two mutants were studied in some detail. In the first one a conserved non-polar residue (Met-44) in the hydrophobic patch was replaced by a lysine. NMR studies of the mutant showed that the overall three-dimensional structure of the protein had not been affected to any great extent by the mutation. This impres-

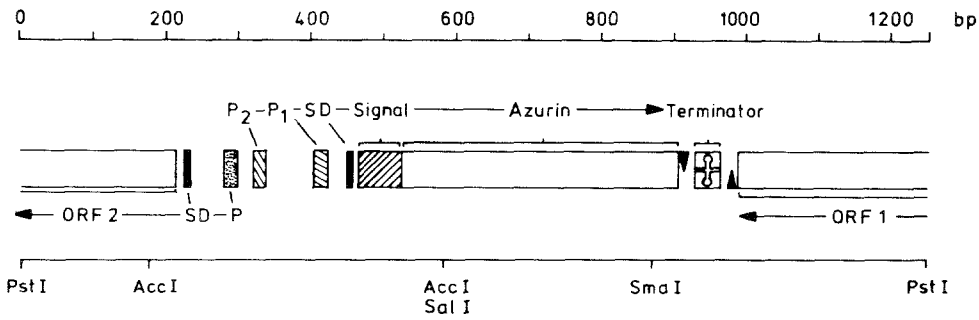

Fig. 5. Organization of the *PstI* fragment on which the azurin gene from *P. aeruginosa* is located. ORF, Open reading frame; P, P_1, P_2, promoters; SD, Shine-Dalgarno sequence; Terminator, *rho*-independent bidirectional terminator; Signal, sequence coding for signal peptide; Azurin, sequence coding for the mature azurin. The scale on top measures the position on the fragment in base pairs. The line underneath indicates the position of a number of endonuclease restriction sites

sion was confirmed by the results of optical and EPR investigations. Nevertheless, and interestingly enough, compared to wild-type azurin, small but distinct changes were seen in the absorbance at 628 nm (decrease of ε_{628} by 10% for the mutant), the EPR parameters ($A_{//}=58$ (wild-type) and 64 cm^{-1} (mutant); $g_{//}=2.257$ (wild-type) and 2.241 (mutant)] and the redox potential (increase of about 50 mV over wild-type). (In passing it is noted that site-directed mutagenesis studies of this type finally provide a means for studying the relation between structure and Cu-site characteristics in more detail than was hitherto possible.)

Contrary to the structural findings, the ESE rate appeared to be strongly affected by the mutation. At pH 5 and 298 K the ESE rate was too slow to be measured by the NMR line-broadening method, and only an upper limit of $<1\times10^3\ M^{-1}\ s^{-1}$ could be established, as opposed to $9.6\times10^5\ M^{-1}\ s^{-1}$ observed for wild-type azurin. That the lower ESE rate is not due to the inactivation of the Cu site as a result of the mutation was demonstrated by the observation that at high pH (pH 9) the ESE rate of the mutant increased to $1\times10^5\ M^{-1}\ s^{-1}$. The pH dependence of the ESE rate has tentatively been ascribed to the protonation of Lys-44 in the mutant.

The second mutant that was investigated carried a His-35→Gln mutation. Also for this mutant only small effects on the three-dimensional structure as compared to the wild type were observed and again small variations in optical and EPR characteristics were observed. This time, however, the ESE rate appeared to be the same as for the wild type over the whole pH range of 5-9. The conclusion of these experiments is that the ESE reaction proceeds by way of the hydrophobic patch, and not via His-35.

Azurin from *A. denitrificans*

Although the azurins from *A. denitrificans* and *P. aeruginosa* differ in their amino acid sequences at no less than 49 positions, their three-dimensional structures are remarkably similar (Ryden and Lundgren 1976; Guss et al. 1986; Adman 1986; Baker 1988). The titration behaviour of His-35, however, is different. This residue titrates with a pK_a of 7.1 in reduced *P. aeruginosa* azurin (5.9 in the oxidized protein) (Ugurbil et al. 1977; Hill and Smith 1979; Rosen et al. 1981; Mitra and Bersohn 1982; Corin et al. 1983), but in the *A. denitrificans* protein no protonation is observed in the pH range 5-9 (Groeneveld et al. 1988). The reason for this is probably that the cleft in the protein structure in which His-35 resides is less accessible for the solvent in the *A. denitrificans* azurin than in the *P. aeruginosa* azurin (Adman 1986; Baker 1988). In the previous section it was seen how a single mutation in the *P. aeruginosa* azurin (in the hydrophobic patch) could have a drastic effect on the ESE rate. It may seem all the more remarkable, therefore, that the 49 amino acid differences between the sequences of the two azurins appear to have no major effect on the ESE rate: at 297 K, pH 6.7, the ESE rate of *A. denitrificans* azurin amounts to $4\times10^5\ M^{-1}\ s^{-1}$ (Groeneveld et al. 1988) compared to *P. aeruginosa* azurin: $9.6\times10^5\ M^{-1}\ s^{-1}$ (Groeneveld and Canters 1988). This becomes understandable, however, when one realizes that the differences in amino-acid sequence have no effect on the hydrophobic character of the surface patch around His-117. The findings once more underline the importance of the hydrophobic patch for the formation of the association complex and the course of the ESE reaction.

The three-dimensional structure of *A. denitrificans* azurin has been reported at a much higher resolution than that of *P. aeruginosa* azurin (0.18 nm cf. 0.27 nm; Adman 1986; Guss et al. 1986; Baker 1988). In principle *A. denitrificans* azurin thus is a better candidate for site-directed mutagenesis than the *P. aeruginosa* azurin. As a first step towards site-directed mutagenesis studies of the *A. denitrificans* azurin the gene coding for the protein has been located on a fragment isolated from a restriction digest of the chromosomal DNA. The gene has subsequently been sequenced. The amino-acid sequence derived from the DNA sequence appears to be at variance with the published sequence at five places. Furthermore the azurin gene is found to code for a preprotein with a signal peptide, like the *P. aeruginosa* azurin. In other respects, however, the gene organisation differs from that found for the *P. aeruginosa* azurin gene. Further details will be reported elsewhere (Hoitink et al. 1990).

Conclusion

The pH effects reported here are varied in nature. In amicyanin the Cu site structure is severely affected at low pH by the protonation of one of the ligand histidines. Concomitantly the redox activity of the protein is abolished, at least as probed by the ESE reaction. It will be interesting to see how pH affects the electron exchange between the amicyanin and its partners methylamine dehydrogenase and cytochrome c_{550}. The pH effects on the three-dimensional structure observed for the *P. aeruginosa* azurin are brought about by structural changes in the second coordination shell of the Cu as a result of the protonation/deprotonation of His-35. The redox activity of the Cu centre itself does not seem to be affected; it remains to be seen to what extent His-35 is involved in ET between the azurin and cytochrome c_{551}. A study of the reaction between cytochrome c_{551} and His-35 mutants, planned for the near future, will hopefully clarify this point. It is clear already that the ESE reaction proceeds through His-117 and that the hydrophobic patch is of paramount importance in the formation of the azurin/azurin encounter complex.

Finally, pH effects on the three-dimensional structure of *A. denitrificans* azurin have not been observed so far. This may be an indication that pH effects on the azurins (when at all present) have no direct physiological implication. Construction of mutants is underway.

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