Properties of *Paracoccus denitrificans* Amicyanin†

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ABSTRACT: *Paracoccus denitrificans* synthesizes an inducible, periplasmic, blue copper protein [Husain, M., & Davidson, V. L. (1985) *J. Biol. Chem. 260*, 14626–14629] that can be classified as an amicyanin on the basis of its ability to accept electrons from methylamine dehydrogenase. The amino acid composition and sequence of the 10 N-terminal residues of this protein have been determined. From these data, it is evident that amicyanin is structurally distinct from azurins as it contains no disulfide bond and an N-terminal sequence that is completely different from the highly conserved N-terminal azurin sequences. Dialysis of reduced amicyanin against potassium cyanide resulted in a nearly quantitative yield of apoamicyanin. Amicyanin and apoamicyanin exhibit fluorescence emission maxima at 314 nm when excited at 280 nm. Addition of 6 M guanidine hydrochloride shifts these emission maxima to 350 nm. The fluorescence intensity of apoamicyanin is 10-fold greater than that of amicyanin. Addition of copper to the apoprotein caused a stoichiometric quenching of fluorescence and restoration of visible absorbance with no concomitant change in absorbance at 280 nm. At least one cysteine residue, which reacts with 5,5′-dithiobis(2-nitrobenzoic acid) in apoamicyanin, does not react in the holoprotein, even in the presence of 6 M guanidine hydrochloride. Reductive and oxidative titrations of amicyanin indicate that it is a one-electron carrier. This amicyanin is also able to accept electrons from the methylamine dehydrogenase isolated from bacterium W3A1, which is taxonomically very different from *P. denitrificans*.

Bacteria and plants contain low molecular weight type I blue copper proteins that function in biological electron transport [reviewed by Fee (1975), Lappin, (1981), Gray & Solomon (1981), and Ryden (1984)]. The plastocyanins and azurins are the best characterized of these proteins. Primary sequence determinations and X-ray crystallographic studies (Coleman et al., 1978; Adman et al., 1978) have contributed to a precise knowledge of their structures and copper binding sites. Recently, another class of blue copper proteins named amicyanins has been identified in methylotrophic bacteria (Tobari & Harada, 1981; Tobari, 1984; Lawton & Anthony, 1985; Husain & Davidson, 1985). These proteins exhibit absorption (Tobari, 1984; Lawton & Anthony, 1985; Hasain & Davidson, 1985) and EPR[1] (Husain & Davidson, 1985) spectra that are characteristic of type I copper proteins. Functionally and physiologically, they are quite distinct from azurins. While a physiological role for amicyanin, that of mediating the transfer of electrons from methylamine dehydrogenase to cytochrome *c*, has been documented, little is known about the physical properties of this class of proteins. The amino acid compositions of a few amicyanins are known (Tobari, 1984; Lawton & Anthony, 1985), but no primary sequence data have been reported, and aside from the absorption and EPR spectra, no information concerning the copper binding site is available.

A blue copper protein, which functionally can be classified as an amicyanin, has been isolated from the facultative autotroph *Paracoccus denitrificans* (Husain & Davidson, 1985). This protein is induced only during growth on methylamine, synthesized to high levels, functions in the periplasmic space, and mediates electron transfer between the periplasmic methylamine dehydrogenase of *P. denitrificans* and cytochrome *c*. This paper extends our initial studies of this protein. The amino acid composition and N-terminal sequence of this protein are reported and compared with those of other blue copper proteins. Significant differences in the primary structure of this amicyanin and the azurins are evident. Apoamicyanin has been prepared, and information on relationships between protein conformation and copper binding has been obtained from the absorption and fluorescence spectra of the apo- and holoproteins and their reactivity with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Some of the redox properties of this amicyanin are described, and the ability of this protein to interact with the methylamine dehydrogenase of bacterium W3A1, a restricted faculative methylotroph (Colby & Zatman, 1975), is documented.

Experimental Procedures

Materials. DTNB and *Pseudomonas aeruginosa* azurin were obtained from Sigma. Ultrapure guanidine hydrochloride was obtain from Schwarz/Mann. All other chemicals were reagent-grade.

Methods. P. denitrificans (ATCC 13543) was grown aerobically at 30 °C in the medium of Kornberg and Morris (1968), supplemented with 0.05% $NaHCO_3$, 0.01% yeast extract, and 0.5% methylamine. Bacterium W3A1 (NCIB 11348) was grown aerobically at 30 °C in the medium of

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PQQ, pyrroloquinoline quinone; EPR, electron paramagnetic resonance; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Table I: Amino Acid Composition of *P. denitrificans* Amicyanin and Comparison with Other Amicyanins and *Ps. aeruginosa* Azurin[a]

	amicyanins				azurin
amino acid	*P. denitrificans*	*Ps.* sp. strain AM1	*Methylomonas* sp. strain J	organism 4025	*Ps. aeruginosa*
Asx	9.2	7	10	18	18
Thr	9.1	8	12	6	10
Ser	4.0	6	9	7	10
Glx	12.0	15	7	5	10
Pro	8.8	8	5	3	4
Gly	9.5	10	13	7	11
Ala	16.5	11	11	8	7
Cys[b]	1.9	1	3	nd[c]	3
Val	13.1	13	10	9	10
Met	6.0	2	5	7	6
Ile	4.4	3	3	2	4
Leu	5.3	3	4	4	10
Ty	5.0	2	4	1	2
Phe	4.9	4	7	11	6
Trp	1.8	1	1	nd	1
Lys	9.8	10	9	10	11
His	6.0	3	5	5	4
Arg	3.2	2	3	3	1
total	129	109	121	nd	128

[a] Comparison compositions from Tobari (1984), Lawton and Anthony (1985), and Ambler and Brown (1967). [b] Determined after performic acid oxidation (Hirs, 1967). [c] Not determined.

Owens and Keddie (1969) supplemented with 0.3% methylamine.

Preparation of amicyanin and methylamine and methanol dehydrogenases from *P. denitrificans* was as described by Husain and Davidson (1985). Preparation of methylamine and methanol dehydrogenases from bacterium W3A1 was as described by Kasprzak and Steenkamp (1983). For preparation of apoamicyanin, 3 mL of 65 μM amicyanin was reduced by addition of excess sodium dithionite, dialyzed for 20 h at 4 °C against 0.1 M Tris-HCl, pH 8.0, containing 0.1 M KCN (Morpurgo et al., 1972; McMillin et al., 1974), and then dialyzed against 0.05 M potassium phosphate, pH 7.1. This procedure resulted in greater than 90% yield of apoamicyanin from the holoprotein.

Reduction of amicyanin by methylamine dehydrogenase was assayed as described by Husain and Davidson (1985). Anaerobic reductive titrations of amicyanin with dithionite were performed according to Williams et al. (1979) in a Thunberg-type cuvette, which was equipped with a gas-tight syringe. Oxidative titrations with ferricyanide were performed aerobically, as reduced amicyanin is stable under these conditions.

Amino acid analyses were performed with a Durram D-500 analyzer. Samples were hydrolyzed at 110 °C in constant-boiling 6 M HCl before (Moore & Stein, 1960) and after performic acid oxidation (Hirs, 1967). Tryptophan was determined spectrophotometrically by the method of Edelhoch (1967). The amino acid composition was calculated on the basis of a M_r of 14 500 (Husain & Davidson, 1985). N-Terminal sequence analysis was performed with a Beckman 890C spinning-cup sequenator, updated with cold trap and microprocessor-based programmer. A dilute (0.1 M) Quadrol program (Beckman no. 012981) was used. Samples were sequenced in the presence of 2 mg of Polybrene (1,5-dimethyl-1,5-diazundecamethylene polymethobromide). The repetitive yield was 96%. Phenylthiohydantoin derivitives of amino acids were analyzed by GLC (Pisano & Bonzert, 1972), TLC (Kulbe, 1974), and HPLC (Bhown et al., 1978). Amino acid residues were confirmed by a positive identification from at least two of these methods.

Absorption spectra were recorded with a Cary 219 spectrophotometer. Fluorescence measurements were made with a Perkin-Elmer MPF-3 fluorescence spectrophotometer. The reaction of DTNB with sulfhydral groups was quantitated from the increase in absorbance at 412 nm with an extinction coefficient of 13 600 M^{-1} cm^{-1} (Means & Feeney, 1971). The concentration of amicyanin was determined from its absorbance at 595 nm with an extinction coefficient of 4600 M^{-1} cm^{-1} (Hausain & Davidson, 1985).

Results

Primary Structure Data. The amino acid composition of *P. denitrificans* amicyanin is given in Table I and compared with the compositions of three other amicyanins for which data are available. Similarities to the compositions of the other three amicyanins are apparent and consistent with their belonging to a functionally distinct family of proteins. Similarities are also apparent, however, to the amino acid composition of azurin. Thus, any attempt to catagorize these proteins solely on the basis of amino acid composition would be invalid. Not reflected by the amino acid compositions is the wide variation in p*I* values exhibited by the amicyanins. The proteins isolated from *P. denitrificans* and organism 4025 are acidic with p*I* values, respectively, of 4.8 (Husain & Davidson, 1985) and 5.3 (Lawton & Anthony, 1985). Those isolated from *Pseudomonas* sp. strain AM1 and *Methylomonas* sp. strain J are basic with p*I* values, respectively, of 9.3 and 7.7 (Tobari, 1984). This variation in p*I* values is probably due to differences in the extent to which the acidic residues of each protein are amidated. Among amicyanins, there is also variation in the numbers of cysteines and tryptophans, residues that are highly conserved in azurins and plastocyanins (Ryden & Lundgren, 1976; Ryden, 1984).

Comparison of the amino acid composition and N-terminal amino acid sequence of *P. denitrificans* amicyanin with that of azurin can provide some insight into the structural features of amicyanin as the details of the three-dimensional structure of *Ps. aeruginosa* azurin are known (Adman et al., 1978). Thus far, each azurin that has been sequenced contains one disulfide bond, linking cysteine residues at positions 3 and 26, and of the blue copper proteins of which structures are known, each employs one cysteine, one methionine, and two histidines as copper ligands (Ryden, 1984). It is not possible for *P. denitrificans* amicyanin to both use cysteine as a copper ligand and possess a disulfide bond because it contains only two cysteines. The N-terminal sequences of azurins are highly

Table II: Sequence of the Ten N-Terminal Residues of *P. denitrificans* Amicyanin and Comparison with N-Terminal Azurin Sequences

amicyanin[a]	
P. denitrificans	Asp-Lys-Ala-Thr-Ile-Pro-Ser-Gln-Ser-Pro-
azurins[b]	
Ps. aeruginosa	Ala-Glu-Cys-Ser-Val-Asp-Ile-Gln-Gly-Asn-
Ps. denitrificans	Ala-Glu-Cys-Ser-Val-Asp-Ile-Gln-Gly-Asn-
Bordetella bronchiseptica	Ala-Glu-Cys-Ser-Val-Asp-Ile-Ala-Gly-Thr-
Alcaligenes sp.	Ala-Glu-Cys-Ser-Val-Asp-Ile-Ala-Gly-Asn-
Ps. fluorescens B-93	Ala-Glu-Cys-Lys-Thr-Thr-Ile-Asp-Ser-Thr-
Ps. fluorescens C-18	Ala-Glu-Cys-Lys-Val-Thr-Val-Asp-Ser-Thr-
Ps. fluorescens D-35	Ala-Glu-Cys-Lys-Val-Asp-Val-Asp-Ser-Thr-
Alcaligenes denitrificans	Ala-Gln-Cys-Glu-Ala-Thr-Ile-Glu-Ser-Asn-
Alcaligenes faecalis	Ala- -Cys-Asp-Val-Ser-Ile-Glu-Gly-Asn-

[a] The methods used to determine and confirm the identities of these amino acids and repetitive yields are given under Experimental Procedures. [b] Azurin sequences are from Ryden (1984).

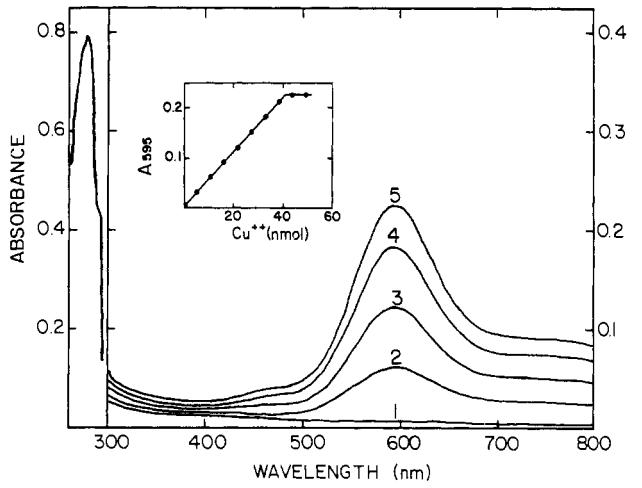

FIGURE 1: Reconstitution of apoamicyanin with $CuSO_4$. A total of 0.8 mL of apoamicyanin (41 nmol) in 50 mM potassium phosphate, pH 7.1, was titrated with a 1.1 mM solution of $CuSO_4$. Spectra were recorded 10 min after the addition of (curve 1) 0, (curve 2) 11, (curve 3) 22, (curve 4) 33, and (curve 5) 44 nmol of Cu^{2+}. The spectra recorded after the addition of intermediate amounts were omitted for clarity. Data for the complete titration are shown in the inset.

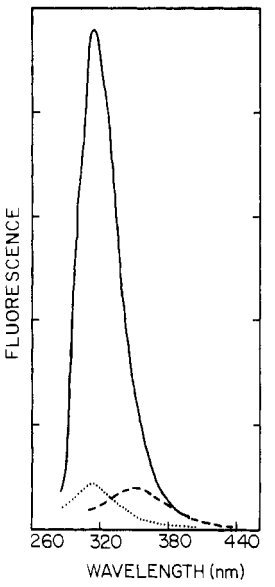

FIGURE 2: Fluorescence spectra of amicyanin and apoamicyanin. Spectra of (—) apoamicyanin and (···) amicyanin in 50 mM potassium phosphate, pH 7.1, and (- -) apoamicyanin and amicyanin in the presence of 6 M guanidine hydrochloride were recorded at 25 °C with an excitation wavelength of 280 nm. The latter two spectra were identical. Each spectrum was recorded with equivalent amounts of protein.

conserved (Ryden & Lundgren, 1976; Ryden, 1984). The N-terminus of the amicyanin, which contains essentially the same number of total amino acids, bears no similarities to those of the azurins (Table II) and contains no cysteine at position 3. Furthermore, the reaction of DTNB with apoamicyanin indicates the presence of free cysteine (discussed below). The absence of a disulfide bond in amicyanin and its lack of identity with the highyl conserved N-terminal sequence of azurins clearly distinguish *P. denitrificans* amicyanin from the family of proteins called azurins on a structural, as well as a physiological, basis.

Absorbance and Fluorescence Properties of Amicyanin and Apoamicyanin. The single tryptophan residue present in *Ps. aeruginosa* azurin has been exploited as a probe of the hydrophobic core of that protein. Analysis of *P. denitrificans* amicyanin for tryptophan by the method of Edelhoch (1967) yielded a value of 1.8 mol/mol of protein. As such, the environment of tryptophan in amicyanin and its relationship to the copper binding site were examined by comparing the absorption spectra and intrinsic fluorescence of amicyanin and apoamicyanin. Apoamicyanin was prepared by a method similar to those used to remove copper from other small blue copper proteins (Morpurgo et al., 1972; McMillin et al., 1974). Removal of copper from the amicyanin resulted in the complete loss of absorbance at 595 nm. This absorbance was fully restored by the readdition of stoichiometric amounts of copper (Figure 1). The reassociation of copper with apoamicyanin, as monitored by the increases in A_{595}, was relatively slow. The maximum change in absorbance caused by the addition of each increment of copper was observed after approximately 10 min. Once the apoprotein was fully titrated with a stoichiometric amount of copper, addition of excess copper caused no further change in absorbance. Dilution of amicyanin into 6 M guanidine hydrochloride resulted in a slow (45-min) bleaching of visible absorbance, presumably due to the slow resolution of copper from the protein. The intrinsic fluorescence of the amicyanin and apoamicyanin was examined in the presence and absence of 6 M guanidine hydrochloride (Figure 2). The fluorescence spectra of amicyanin and apoamicyanin showed excitation maxima at 280 nm (data not shown) and emission maxima at 314 nm (Figure 2). This can be compared to a fluorescence maximum of azurin of 308 nm when excited at 280 nm (Finazzi-Agro et al., 1970). The emission maxima of amicyanin and apoamicyanin in the presence of 6 M guanidine hydrochloride were shifted to 350 nm, typical of tryptophan fluorescence in an aqueous environment. The fluorescence intensity at 314 nm of the native apoamicyanin is approximately 10-fold greater than that of the holoprotein or denatured proteins. Quenching of fluorescence of the apoprotein to levels of intensity observed in the holoprotein can be achieved by addition of stoichiometric amounts of copper to the apoprotein (data not shown). The addition of copper to the apoprotein had no effect on the absorbance of

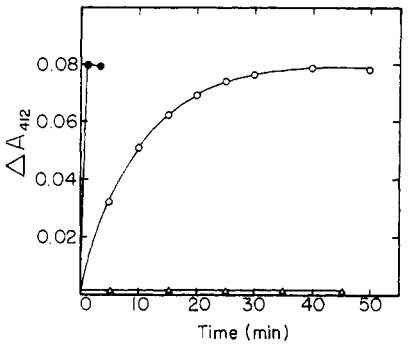

FIGURE 3: Reaction of amicyanin and apoamicyanin with DTNB. Amicyanin (5.9 μM) and apoamicyanin (5.9 μM) were incubated at 25 °C in 50 mM Tris-HCl, pH 8.0, with 250 mM DTNB. Reference cuvettes contained only buffer. The reaction was started by the addition of DTNB to both cuvettes. (△) Amicyanin; (○) apoamicyanin; (●) apoamicyanin in the presence of 6 M guanidine hydrochloride.

the protein at 280 nm (Figure 1). Addition of copper in excess of stoichiometric amounts caused no further quenching of fluorescence.

Accessibility of Cysteine in Amicyanin and Apoamicyanin. Analysis of the cysteine content of amicyanin after performic acid oxidation indicated the presence of 1.9 mol of cysteine/mol of protein. The accessibility to the external medium of cysteine residues in amicyanin and apoamicyanin was probed with DTNB (Figure 3). No increase in A_{412} was observed upon incubation of amicyanin with DTNB. Incubation of apoamicyanin with DTNB resulted in a slow increase in A_{412} corresponding to the reaction of 1.0 mol of cysteine/mol of protein. Incubation of apoamicyanin with DTNB in the presence of 6 M guanidine hydrochloride resulted in an immediate increase in A_{412} corresponding to the reaction of 1.0 mol of cysteine/mol of protein. No such immediate reaction was observed with amicyanin in the presence of 6 M guanidine hydrochloride. Under these conditions, reaction with DTNB was observed only after an incubation time long enough to cause loss of visible absorbance (data not shown), which presumably indicated the removal of copper from the protein. The inability to observe the reaction of a second cysteine with DTNB is surprising. It may be due to prior oxidation of this residue during or subsequent to the preparation of the apoprotein. All procedures were performed under aerobic conditions. Thus, while copper was removed from the holoprotein as the Cu(I) complex, once resolved from the native protein, it could conceivably have been converted to Cu(II) and facilitated the autooxidation of an unprotected cysteine residue (Cavallini et al., 1969; Jocelyn, 1972).

Redox Properties of Amicyanin. An anaerobic reductive titration of *P. denitrificans* amicyanin with sodium dithionite ($E^{\circ\prime}$ = −530 mV) was performed and monitored by changes in A_{595} (Figure 4A). Full reduction, as judged by the complete bleaching of visible absorbance, required 0.5 mol of dithionite/mol of amicyanin. Back-titration of the fully reduced amicyanin consumed 1.0 mol of ferricyanide ($E^{\circ\prime}$ = 430 mV) per mole of protein (Figure 4B). These data indicate that *P. denitrificans* amicyanin is a one-electron carrier.

Reactivity of Amicyanin and Azurin with Methylamine and Methanol Dehydrogenases. P. denitrificans amicyanin has been shown to accept electrons from the methylamine dehydrogenase isolated from that organism (Husain & Davidson, 1985). This enzyme, which functions in the periplasmic space (Husain & Davidson, 1985) and is believed to contain a pyrroloquinoline quinone (PQQ) prosthetic group (Duine et al., 1981), is commonly found in methylotrophic bacteria

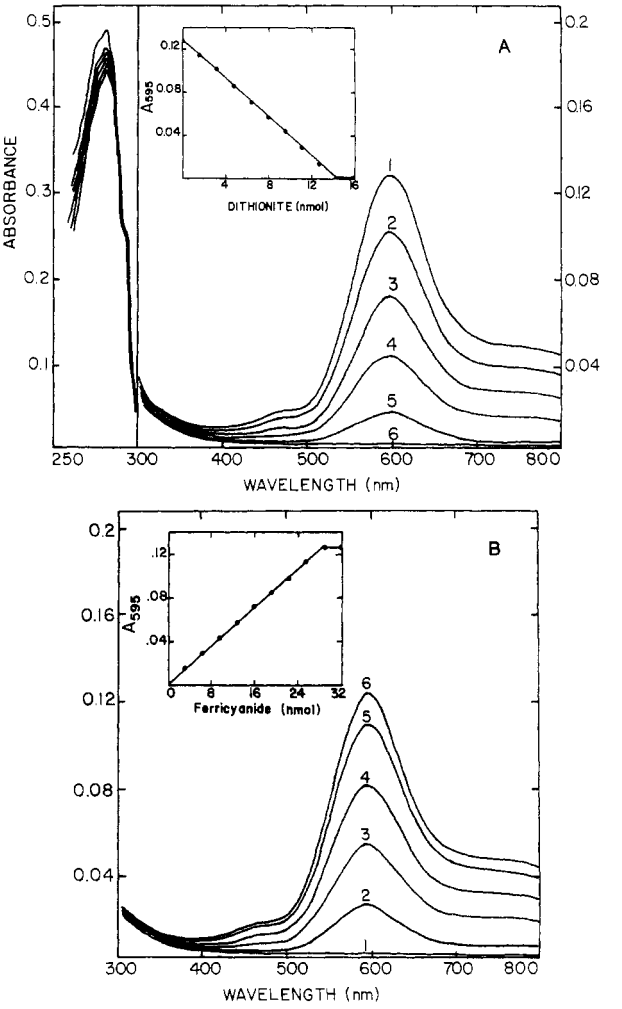

FIGURE 4: (A) Dithionite titration of amicyanin. A total of 1 mL of amicyanin (28 nmol) in 50 mM potassium phosphate, pH 7.1, was deoxygenated and titrated with an anaerobic solution of 1.6 mM dithionite at 25 °C. Spectra were recorded after addition of (curve 1) 0, (curve 2) 3.2, (curve 3) 6.4, (curve 4) 9.6, (curve 5) 12.8, and (curve 6) 14.3 nmol of dithionite. The spectra recorded after the addition of intermediate amounts were omitted for clarity. Data for the complete titration are shown in the inset. A total of 14.1 nmol of dithionite was consumed in the complete reduction. (B) Reoxidation of amicyanin by ferricyanide. The reduced amicyanin sample was transfered to a 1-mL cuvette, exposed to air to oxidize any excess dithionite, and titrated with a solution of 1.6 mM potassium ferricyanide. Spectra were recorded after addition of (curve 1) 0, (curve 2) 6.4, (curve 3) 12.8, (curve 4) 19.2, (curve 5) 25.6, and (curve 6) 32.2 nmol of ferricyanide. The spectra recorded after the addition of intermediate amounts were omitted for clarity. Data for the complete titration are shown in the inset. A total of 28.3 nmol of ferricyanide was consumed in the complete oxidation.

(Anthony, 1982). The ability of *P. denitrificans* amicyanin to accept electrons from the analogous periplasmic, PQQ-containing enzyme isolated from bacterium W3A1 was examined. This bacterium is taxonomically very different from *P. denitrificans* (Colby & Zatman, 1975; Jenkins et al., 1984; Davidson, 1985). While bacterium W3A1 methylamine dehydrogenase is functionally similar to the *P. denitrificans* enzyme, it exhibits significant physical differences. The *P. denitrificans* enzyme is an acidic protein, whereas the enzyme isolated from bacterium W3A1 is a basic protein, and the large subunit of the *P. denitrificans* enzyme is much larger than that of the other enzyme (unpublished results). Despite these differences, *P. denitrificans* amicyanin readily accepts electrons from each of these methylamine dehydrogenases. Addition of methylamine to amicyanin in the presence of catalytic

amounts of either methylamine dehydrogenase resulted in the rapid disappearance of A_{595}. *Ps. aeruginosa* azurin did not function effectively as an electron acceptor for methylamine dehydrogenase. Under identical experimental conditions, reduction of azurin by methylamine dehydrogenase was observed, but with a half-time of reaction greater than 1 h. *P. denitrificans* amicyanin was not reduced by the periplasmic, PQQ-containing methanol dehydrogenase isolated from bacterium W3A1, consistent with the previous observation that *P. denitrificans* methanol dehydrogenase did not donate electrons to amicyanin (Husain & Davidson, 1985).

Discussion

The recently discovered amicyanins have been catagorized as such on the basis of a single physiological function. Beyond this, there is little information available that distinguishes amicyanins from other well-characterized small blue copper proteins, such as azurin and plastocyanin. *P. denitrificans* amicyanin exhibits absorption and EPR spectra typical of type I copper proteins and similar to those of azurins and plastocyanins (Husain & Davidson, 1985). Previous studies of *P. denitrificans* amicyanin (Husain & Davidson, 1985) have provided certain physiological criteria that clearly distinguish it from azurins: it mediates the transfer of electrons between methylamine dehydrogenase and cytochrome *c*, is an inducible protein, and functions in the periplasmic space. This paper provides physical evidence that clearly distinguishes this amicyanin from azurins and provides physical criteria for classifying proteins as amicyanins. The N-terminal sequences of azurins and plastocyanins are highly conserved (Ryden & Lundgren, 1976). The N-terminal sequence of *P. denitrificans* amicyanin resembles neither. When sequence data for other amicyanins become available, a comparison with the data presented in this paper will indicate whether or not amicyanins also possess a highly conserved N-terminal domain.

The fluorescence emission maximum of amicyanin of 314 nm, when excited at 280 nm, resembles that of azurin of 308 nm (Finazzi-Agro et al., 1970) and indicates that tryptophan in amicyanin, as in azurin, residues in an extremely hydrophobic environment. The quenching of apoamicyanin fluorescence by readdition of copper is also consistent with previous observations that copper binding results in partial quenching of fluorescence of apoazurin (Finazzi-Agro et al., 1970), apostellacyanin (Morpurgo et al., 1972), and apoplastocyanin (Graziani et al., 1974). The 90% quenching of apoamicyanin fluorescence caused by readdition of copper is even more dramatic than the 70% quenching of apoazurin fluorescence by copper (Finazzi-Agro et al., 1970) and suggests that, as in azurin, the tryptophan residues of amicyanin reside in a very hydrophobic environment in close proximity to the bound copper. That a cysteine residue is accessible to DTNB only in the apoamicyanin and reacts much more rapidly in the presence of 6 M quanidine hydrochloride is consistent with it providing a ligand for copper, as is the case for azurin and plastocyanin. Thus, it appears that while the structural details of amicyanin and azurin differ, the residues that comprise the environment of the copper binding site may be conserved. Determination of the complete primary sequence and X-ray crystallographic data are needed to make a definitive comparison of the structural properties of amicyanin to those of other blue copper proteins.

The ability of *P. denitrificans* amicyanin to interact with the methylamine dehydrogenase of a taxonomically diverse organism, bacterium W3A1, emphasizes the specificity of amicyanin for this enzyme and implies a critical role for this protein in a primary oxidation pathway of methylotrophic bacteria. The observation that amicyanin can interact with both the acidic methylamine dehydrogenase of *P. denitrificans* and the basic enzyme from bacterium W3A1 and that the amicyanins characterized thus far exhibit a wide variation in pI values suggest that surface charge does not play an important role in the interaction of amicyanin with methylamine dehydrogenase. Also consistent with this interpretation is the observation that *P. dentrificans* amicyanin, which has a pI value of 4.8, does not bind to DEAE-cellulose at neutral pH (Husain & Davidson, 1985).

Methylamine dehydrogenase, the physiological electron donor for amicyanin, catalyzes the oxidation of methylamine to formaldehyde and ammonia. Each of the two PQQ cofactors present in methylamine dehydrogenase has a two-electron capacity (Kenny & McIntire, 1983). Thus, the enzyme is capable of transferring four electrons. The actual catalytic mechanism of PQQ enzymes, including methylamine dehydrogenase, is unclear. This paper confirms that amicyanin, the physiological electron acceptor of methylamine dehydrogenase, is a one-electron carrier. The mechanism of its interaction with methylamine dehydrogenase is also unknown. Hopefully, the characterization of *P. denitrificans* amicyanin will allow for studies that will detail not only the mechanisms of copper-containing proteins but also the kinetic properties of PQQ enzymes, such as methylamine dehydrogenase.

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Registry No. Cu, 7440-50-8; L-cysteine, 52-90-4; methylamine dehydrogenase, 60496-14-2.

References

Adman, E. T., Stenkamp, R. E., Siecker, L. C., & Jensen, L. H. (1978) *J. Mol. Biol. 123*, 35–47.

Ambler, R. P., & Brown, L. H. (1967) *Biochem. J. 104*, 784–825.

Anthony, C. (1982) *The Biochemistry of Methylotrophs*, Academic, London.

Bhown, A. S., Mole, J. E., Weissinger, A., & Bennett, J. C. (1978) *J. Chromatogr. 148*, 532–535.

Cavallini, D., De Marco, C., Dupre, S., & Rotilio, G. (1969) *Arch. Biochem. Biophys. 130*, 354–361.

Colby, J., & Zatman, L. J. (1975) *Biochem. J. 148*, 505–511.

Coleman, P. M., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M., & Venkatappa, M. P. (1978) *Nature (London) 272*, 319–324.

Davidson, V. L. (1985) *J. Bacteriol. 164*, 941–943.

Duine, J. A., Frank, J., Jr., & Westerling, J. (1981) *Eur. J. Biochem. 118*, 395–399.

Edelhoch, H. (1967) *Biochemistry 6*, 1948–1954.

Fee, J. A. (1975) *Struct. Bonding (Berlin) 23*, 1–60.

Finazzi-Agro, A., Rotilio, G., Avigliamo, L., Guerrieri, P., Boffi, V., & Mondovi, B. (1970) *Biochemistry 9*, 2009–2014.

Gray, H. B., & Solomon, E. I. (1981) in *Copper Proteins* (Spiro, T. G., Ed.) pp 1–39, Wiley, New York.

Graziani, M. T., Finazzi-Agro, A., Rotilio, G., Barra, D., & Mondovi, B. (1974) *Biochemistry 13*, 804–808.

Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 59–62.

Husain, M., & Davidson, V. L. (1985) *J. Biol. Chem. 260*, 14626–14629.

Jenkins, O., Byrom, D., & Jones, D. (1984) in *Microbial Growth on C_1 Compounds* (Crawford, R. L., & Hanson, R. S., Eds.) pp 255–261, American Society for Microbiology, Washington, DC.

Jocelyn, P. C. (1972) in *Biochemistry of the SH Group*, pp 94–115, Academic, New York.
Kasprzak, A. A., & Steenkamp, D. J. (1983) *J. Bacteriol. 156*, 348–353.
Kenny, W. C., & McIntire, W. (1983) *Biochemistry 22*, 3858–3868.
Kornberg, H. L., & Morris, J. G. (1968) *Biochem. J. 95*, 577–586.
Kulbe, K. D. (1974) *Anal. Biochem. 59*, 564–573.
Lappin, A. G. (1981) *Met. Ions Biol. Syst. 13*, 15–71.
Lawton, S. A., & Anthony, C. (1985) *Biochem. J. 228*, 719–726.
McMillin, D. R., Rosenberg, R. C., & Gray, H. B. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4760–4762.
Means, G. E., & Feeney, R. E. (1971) in *Chemical Modification of Proteins*, p 220, Holden-Day, San Francisco.
Moore, S., & Stein, W. H. (1960) *Methods Enzymol. 6*, 819–831.
Morpurgo, L., Finazzi-Agro, A., Rotilio, G., & Mondovi, B. (1972) *Biochim. Biophys. Acta 271*, 292–299.
Owens, J. D., & Keddie, R. M. (1969) *J. Appl. Bacteriol. 32*, 338–347.
Pisano, J. J., & Bonzert, T. J. (1972) *Anal. Biochem. 45*, 43–59.
Ryden, L. (1984) in *Copper Proteins and Copper Enzymes* (Lontie, R., Ed.) Vol. 1, pp 157–182, CRC Press, Boca Raton, FL.
Ryden, L., & Lundgren, J.-O. (1976) *Nature (London) 261*, 344–346.
Tobari, J. (1984) in *Microbial Growth on C_1 Compounds* (Crawford, R. L., & Hanson, R. S., Eds.) pp 106–112, American Society for Microbiology, Washington, DC.
Tobari, J., & Harada, Y. (1981) *Biochem. Biophys. Res. Commun. 101*, 502–508.
Williams, C. H., Jr., Arscott, L. D., Matthews, R. G., Thorpe, C., & Willkinson, K. D. (1979) *Methods Enzymol. 62*, 185–198.

Spectral Properties of Nitric Oxide Complexes of Cytochrome *c'* from *Alcaligenes* sp. NCIB 11015†

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ABSTRACT: For the purpose of clarifying the stereochemistry of the heme environment and the electronic structure of the heme iron in cytochrome *c'* from *Alcaligenes* sp. NCIB 11015, the spectral properties of the nitric oxide (^{14}NO and ^{15}NO) derivatives of cytochrome *c'* have been investigated. The electron paramagnetic resonance (EPR) and electronic spectra vary with the change of pH either at room temperature or at 77 K. The results of EPR and electronic spectroscopies on NO–*Alcaligenes* cytochrome *c'* at physiological pH indicate that the heme iron to histidine (Fe–N_ϵ) bond of cytochrome *c'* is very weak and is cleaved upon the coordination of a nitrosyl group. From a comparison of the electronic spectra for NO–cytochrome *c'* from various species, the decreasing order of the Fe–N_ϵ bond strength among species has been considered to be *Rhodopseudomonas palustris* > *Rhodospirillum rubrum* > *Alcaligenes*. The reaction of NO with *Alcaligenes* ferric cytochrome *c'* results in the formation of NO–ferrous cytochrome *c'*, which is interpreted on the basis of a reductive nitrosylation.

Nitric oxide is coordinated as a heme sixth ligand to heme iron in hemoproteins, and the nitrosyl derivatives of hemoproteins have spectral properties that vary with the environmental difference of the heme and the electronic structure of the heme iron. Since nitric oxide has an unpaired electron, the nitrosylhemoproteins have been extensively studied by EPR[1] spectroscopy. Studies on the nitrosylhemoproteins have been carried out also by the use of electronic, IR, resonance Raman, MCD, and NMR spectroscopies [Yoshimura (1983a) and references cited therein]. On the other hand, studies on the model complex nitrosyl(porphyrinato)iron(II) under various conditions have given significant clues to understanding

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[1] Abbreviations: EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism; CD, circular dichroism; IR, infrared; NMR, nuclear magnetic resonance; *Alcaligenes*, *Alcaligenes* sp. NCIB 11015; *R.*, *Rhodospirillum*; *Rps.*, *Rhodopseudomonas*; *Chromatium*, *Chromatium vinosum*; *A.*, *Azotobacter*; cyt, cytochrome; Hb, hemoglobin; Mb, myoglobin; CCP, cytochrome *c* peroxidase; IDO, indoleamine 2,3-dioxygenase; PPIX, dianion of protoporphyrin IX; PPIXDME, dianion of protoporphyrin IX dimethyl ester; TPP, dianion of *meso*-tetraphenylporphyrin; SDS, sodium dodecyl sulfate; IHP, inositol hexakis(phosphate); NMeIm, 1-methylimidazole; Tris, tris(hydroxymethyl)aminomethane.

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