[42] Blue Copper Proteins Involved in Methanol and Methylamine Oxidation

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Introduction

The majority of methylotrophic bacteria, when grown on methylamine as the sole source of carbon and energy, synthesize a specific quinoprotein, methylamine dehydrogenase, which is located in the periplasm, together with its specific electron acceptor, a blue copper protein or cupredoxin, called amicyanin.[1-3] Although amicyanin is the best (usually only) electron acceptor for methylamine dehydrogenase, it is not detectable in all methylotrophs growing on methylamine by way of this enzyme. The difficulty of determining whether amicyanin is essential for methylamine oxidation is due to large variations in amounts of amicyanin found in those bacteria that do produce it, variations in amounts of blue copper proteins brought about by varying copper or iron concentrations during growth, and variations in the ease of dissociation of copper from the proteins when studied *in vitro*. In some methylotrophs it is possible that cytochrome *c* is able to replace amicyanin, but the extent to which this occurs *in vivo* is uncertain.[4-9]

Provided sufficient copper and iron are included in the growth medium, a second blue copper protein (or cupredoxin) is also synthesized. This is usually called azurin, or pseudoazurin because it has no function in reduction of nitrite and does not necessarily have a primary sequence similar to that of other azurins. It has no known function, but it is probably able to replace cytochrome *c* in mediating the oxidation of amicyanin by oxidase, thus leading to a complete methylamine oxidase electron-transport chain in which soluble cytochromes are completely replaced by blue copper proteins.[8,9] Although azurin cannot replace cytochrome c_L as elec-

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[3] C. Anthony, *Antonie van Leeuwenhoek* **56,** 13 (1989).
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[6] S. A. Lawton and C. Anthony, *J. Gen. Microbiol.* **131,** 2165 (1985).
[7] S. A. Lawton and C. Anthony, *Biochem. J.* **228,** 719 (1985).
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[9] K. A. Auton and C. Anthony, *J. Gen. Microbiol.* **135,** 1923 (1989).

tron acceptor for methanol dehydrogenase, during growth of some organisms on methanol azurin may be present at a sufficiently high level to mediate the oxidation of cytochrome c_L by the oxidase.[8,9]

Assay of Blue Copper Proteins from Absorption Spectra

Concentrations of amicyanin and azurin are determined from the visible absorption spectra of protein oxidized with a few grains of potassium ferricyanide; the reference cuvette contains water. The oxidized proteins have characteristic broad absorption bands in the visible region, with peaks between 590 and 630 nm. Concentrations can be calculated from the appropriate extinction coefficients. If these are not known it is convenient to assume a value of 4 mM^{-1} cm^{-1} (1 μmol in 1 ml has an absorption of 4.0; an absorption of 1.0 is due to 250 nmol). After the blue copper proteins have been purified and extinction coefficients and relative proportions of each protein determined, then the concentrations of each blue copper protein can be determined in crude extracts (see Table I).

Assay of Blue Copper Proteins as Electron Acceptor for Methylamine Dehydrogenase

Reagents

MOPS–KOH buffer, 0.1 *M*, pH 7.0
Methylamine hydrochloride, 0.1 *M*, adjusted to pH 7.0
Methylamine dehydrogenase, 150 n*M*

Procedure and Units. The reaction is measured in a 1-ml spectrophotometer cuvette at 20°. The 1-ml reaction mixture contains 0.25 ml MOPS buffer, 0.1 ml methylamine dehydrogenase, and electron acceptor (blue copper protein) sufficient to give an absorption in the oxidized form of at least 0.1. Methylamine (0.1 ml) is added to start the reaction. Absorption is measured at the wavelength maxima of the protein being studied. One unit of methylamine dehydrogenase reduces 1 μmol of amicyanin per minute (see above for calculation). In this assay, 15 n*M* methylamine dehydrogenase (15 pmol in 1 ml) catalyzes the reduction of about 30 nmol of amicyanin per minute or about 1 nmol of horse heart cytochrome *c* per minute, or about 1.5 nmol oxygen in the dye-linked assay.

Purification of Blue Copper Proteins from Organism 4025

The purification presented below is for the blue copper proteins from an obligate methylotroph, similar to *Methylophilus methylotrophus,* called

TABLE I
PURIFICATION OF AMICYANIN AND AZURIN FROM ORGANISM 4025

Purification step	Volume (ml)	Copper protein (nmol/mg protein)	Copper protein (total, μmol)	Yield (%)	Purification (-fold)
Amicyanin					
Crude extract	80	1.5	5.47	100	1
DEAE-cellulose	83	6.0	4.44	69	3.9
Sephadex G-150	135	33.9	3.07	56	22.2
Mono S	77	76.5	2.44	45	50.0
Mono Q	83	96.8	2.33	43	63.3
Axurin					
Crude extract	150	0.083	0.75	100	1
DEAE-cellulose	100	0.91	0.50	67	10.9
Sephadex G-150	75	2.37	0.40	53	28.5
CM-cellulose	84	17.26	0.31	41	208.0
Mono S	92	81.3	0.29	32	978.6

organism 4025. It is unusual in requiring relatively high copper concentrations for growth on methylamine, during which exceptionally large amounts of both amicyanin and azurin are synthesized. Iron must also be added to growth medium because blue copper proteins are not synthesized in its absence.[9] The methods presented here are probably suitable for other methylotrophs, but, for convenience, references to alternative methods for use with other important methylotrophs are given: *Methylobacterium extorquens* AM1, the methylotroph from which amicyanin was first isolated,[10] and *Paracoccus denitrificans,* in which a convenient starting point can be the periplasmic fraction.[11-13] It should be noted that some blue copper proteins lose copper rather easily; this can be prevented by including 1 μM $CuSO_4$ in purification buffers.

Growth of Organism 4025. Bacteria are grown aerobically in 18-liter batch cultures at 30° on methylamine (0.5%, w/v) for preparation of amicyanin or on methanol (1%, v/v) for preparation of azurin. Growth on methanol leads to production of azurin only and so avoids the problem of extremely high concentrations of amicyanin. The purification methods can be used, however, for both proteins from methylamine-grown bacteria.

The growth medium is that previously described,[14] with the following trace element solution added to give a final concentration in the growth

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medium of 1 mg/liter. The trace element solution contains the following (g/liter): $FeSO_4 \cdot 2H_2O$, 0.2; H_3BO_3, 0.003; $MnSO_4 \cdot 4H_2O$, 0.02; $ZnSO_4 \cdot 7H_2O$, 0.02; Na_2MoO_4, 0.004; $CaCl_2 \cdot 2H_2O$, 0.53; $CoCl_2 \cdot 6H_2O$, 0.004. $CuSO_4 \cdot 5H_2O$ (5 mg per liter) is added separately. The growth medium described here is that used for the purifications described below. Alternative methods for growth of the organism in continuous culture have been described elsewhere.[9]

After harvesting by centrifugation, bacteria are washed twice at 4° in 20 m*M* Tris-HCl buffer (pH 8.0), suspended in the same buffer (100 g wet weight in 270 ml), and disrupted by sonication for 10 min in 1-min periods. Whole bacteria and debris are removed by centrifugation at 6000 *g* for 10 min, and membranes are removed by centrifugation at 138,000 *g* for 1 hr.

Purification of Amicyanin, Azurin, Methylamine Dehydrogenase, and c-Type Cytochromes from Methylamine-Grown Bacteria. The crude extract is passed down a column of DEAE-cellulose (5 × 23 cm) equilibrated with 20 m*M* Tris-HCl buffer (pH 8.0) containing 1.2 μM $CuSO_4$. Methylamine dehydrogenase, amicyanin, azurin, and cytochrome c_H are not adsorbed. The fractions containing these proteins are pooled and concentrated under nitrogen on an Amicon (Danvers, MA) YM2 filter and subjected to gel filtration on a column (4 × 84 cm) of Sephadex G-150 equilibrated with 20 m*M* Tris-HCl (pH 8.0) which separates the dehydrogenase from the smaller electron-transport proteins. The fractions containing these proteins are pooled and dialyzed against 10 m*M* MES–KOH buffer (pH 5.5), concentrated, and subjected to cation-exchange chromatography on a Pharmacia (Piscataway, NJ) fast liquid protein chromatography (FPLC) Mono S column (1 ml) equilibrated with the same buffer.

Amicyanin is not adsorbed to the Mono S column. It is dialyzed against 10 m*M* MOPS–KOH and subjected to anion-exchange chromatography on a Pharmacia Mono Q column equilibrated with the same buffer. Amicyanin is eluted at 300 m*M* NaCl in a gradient of 0–1. 0 *M* NaCl in the same buffer. The pure amicyanin is dialyzed against the same MOPS buffer and stored at −20°.

Methylamine dehydrogenase from the initial gel filtration column is dialyzed against 10 m*M* potassium phosphate buffer (pH 7.0), applied to a column (5 × 14 cm) of CM-cellulose equilibrated with the same buffer, and eluted (at 55–65 m*M* phosphate) by using a linear gradient of 10–100 m*M* potassium phosphate buffer (pH 7.0). The pure dehydrogenase is dialyzed against 10 m*M* MOPS–KOH buffer (pH 7.0) and stored at −20°. The specific activity of the pure protein is 0.67 μmol of oxygen consumed per minute per milligram protein in the published dye-linked assay system;[7] the yield is about 50%.

Cytochrome c_L is eluted from the first DEAE-cellulose column with a

linear gradient of 20–300 m*M* Tris-HCl buffer (pH 8.0). It is concentrated under nitrogen on an Amicon YM2 filter and partially purified by gel filtration on columns (2.5 × 76 cm) of Sephadex G-50, followed by Sephadex G-150, both equilibrated with 20 m*M* MOPS–KOH buffer (pH 7.0).

Azurin (containing cytochrome c_H) is eluted from the Mono S column at 310 m*M* NaCl by using a linear gradient of 0–1.0 *M* NaCl in 10 m*M* MES–KOH buffer (pH 5.5). The azurin produced by this method is 98% pure, the sole contaminant being cytochrome c_H, which can be removed by further runs through the Mono S column.

Purification of Azurin from Organism 4025 Grown on Methanol. The purification of azurin from methanol-grown organism 4025 has the advantage that there is no possibility of contamination with amicyanin, which is not induced during growth on methanol. Crude extract, prepared as described above, is subjected to anion-exchange chromatography on a column (6 × 15 cm) of DEAE-cellulose and gel filtration on Sephadex G-150 (4 × 84 cm) as described for amicyanin purification (above). Fractions containing azurin are dialyzed against 10 m*M* potassium phosphate buffer (pH 6.0) followed by cation-exchange chromatography on a column (6 × 6 cm) of CM-cellulose equilibrated in the same buffer. Azurin is eluted (at 40–50 m*M*) by using a linear gradient of 10–100 m*M* potassium phosphate buffer (pH 6.0). It is dialyzed against 10 m*M* MES–KOH buffer (pH 5.5) and purified to homogeneity on a Pharmacia Mono S column as described above. It is dialyzed against 10 m*M* MOPS–KOH buffer (pH 7.0) and stored at −17°.

Properties of Blue Copper Proteins from Organism 4025[6-8]

Amicyanin. Amicyanin has a molecular weight of 11,500, as measured by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). It has an isoelectric point of 5.3 (isoelectric focusing), which is markedly lower than those of amicyanins from *Methylobacterium extorquens* AM1 (p*I* 9.3).[1] It has a midpoint redox potential at pH 7.0 of +294 mV. In the oxidized state it has an absorption maximum at 620 nm with an extinction coefficient of 3.8 mM^{-1} cm^{-1}. It reacts as electron acceptor with methylamine dehydrogenase and with both cytochrome *c* and azurin but not with the cytochrome oxidase of organism 4025 (a cytochrome *co*).[8]

Azurin. Azurin has a molecular weight of 12,500 (measured by SDS–PAGE) and an isoelectric point of 9.4 (by isoelectric focusing), which is identical to that of azurin from *Methylobacterium extorquens* AM1.[1] It has a midpoint redox potential at pH 7.0 of +323 mV. In the oxidized state it

has an absorption maximum at 620 nm with an extinction coefficient of 16 mM^{-1} cm^{-1}, which is about 4 times higher than that of most other blue copper proteins. Azurin is not an electron acceptor for methylamine dehydrogenase, but it is able to react with amicyanin, cytochrome *c*, and the oxidase (cytochrome *co*).