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AMICYANIN: AN ELECTRON ACCEPTOR OF METHYLAMINE DEHYDROGENASE

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SUMMARY: A type I blue copper protein, "amicyanin", was purified from a cell-free extract of methylamine-grown *Pseudomonas* AM1. It was found that amicyanin is able to serve as a primary electron acceptor of methylamine dehydrogenase. Amicyanin was reduced by the addition of both methylamine dehydrogenase and methylamine. Cytochromes *c* could not be directly reduced but could be reduced with the addition of amicyanin. The results strongly suggest that amicyanin participates as an electron carrier between methylamine dehydrogenase and cytochrome *c* in the electron transport chain of the methylamine-grown cell.

Some obligate and facultative methylotrophs can grow on methylamine as the sole carbon source. The first step of methylamine metabolism in these methylotrophs is initiated by methylamine dehydrogenase [primary amine: (acceptor) oxidoreductase (deaminating) EC 1.4.9.3]. This enzyme has been purified from *Pseudomonas* AM1 (1-3), *Methylomonas methylovora* (4), and *Methylomonas* J (5) and its properties have been studied (1-12). The prosthetic group and electron acceptor of this enzyme, however, remain obscure.

During the course of this study, we found that a component which can serve as an electron acceptor of methylamine dehydrogenase is present in the cell-free extract of methylamine-grown *Pseudomonas* AM1. After purification, this component was found to be a type I blue copper protein, which we named "amicyanin". This paper briefly describes the properties of amicyanin.

MATERIALS AND METHODS

Chemicals Cytochrome *c*-551 (I) and cytochrome *c*-551 (II) were purified from methanol-grown *Methylomonas* J (13). All chemicals were purchased from commercial sources.

Abbreviations: DCPIP, 2,6-dichlorophenol indophenol; PMS, phenazine methosulfate; SDS, sodium dodecyl sulfate.

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Bacterial growth *Pseudomonas* AM1 was grown on a medium (14) supplemented with $CuSO_4 \cdot 5H_2O$, 0.25 mg per liter containing 0.5% methylamine hydrochloride (3).

Methylamine dehydrogenase Methylamine dehydrogenase was purified from methylamine-grown *Pseudomonas* AM1 (3) and the enzyme activity was measured by PMS-DCPIP assay (1).

Amicyanin assay The ability of amicyanin to serve as an electron acceptor of methylamine dehydrogenase was measured using a method similar to the PMS-DCPIP assay (1), except that the reaction mixture contained 5 mM K-phosphate buffer, pH 7.0, and an appropriate amount of amicyanin, instead of 100 mM Na-phosphate buffer, pH 7.5, and PMS. Reaction was started by the addition of the enzyme (0.02 unit) and the reduction rate at 600 nm was spectrophotometrically recorded. The concentration of the purified amicyanin can also be estimated by its absorbance at 596 nm using a millimolar extinction coefficient of 4.5 $mM^{-1}cm^{-1}$, as will be described in RESULTS.

Other methods Molecular weight was determined by SDS-polyacrylamide gel electrophoresis (15) and Sephadex G-75 gel chromatography (16). Protein concentration was determined by the method of Lowry *et al.* (17). The concentration of cytochromes *c* was determined as pyridine ferrohemochrome (18). Optical measurements of the visible-ultraviolet absorption spectrum and the atomic absorption spectrum, and amino acid analysis were performed as described previously (5).

Amicyanin and cytochromes *c* The cell-free extract of methylamine-grown *Pseudomonas* AM1 was fractionated with solid ammonium sulfate (50-95% saturation). After centrifugation, the precipitates were dissolved in a small volume of 10 mM K-phosphate buffer, pH 7.0, and dialysed against the same buffer. The dialysed solution was passed through a TEAE-cellulose column equilibrated with the same buffer. Cytochrome c_L remained on the top of the column as a red band. The unabsorbed solution containing amicyanin, cytochrome c_H and methylamine dehydrogenase was applied to a hydroxyl apatite column equilibrated with the same buffer, then eluted with a linear gradient of a 10 mM - 500 mM phosphate buffer, pH 7.0. The fractions containing amicyanin were concentrated by ammonium sulfate precipitation (90% saturation) and rechromatographed on a hydroxyl apatite column. Amicyanin was eluted with a linear gradient of a 10 mM - 150 mM phosphate buffer, pH 7.0. Eluted fractions containing amicyanin were concentrated to a small volume by freeze-drying. The concentrated solution was applied to a Sephadex G-75 column. SDS-polyacrylamide gel electrophoresis showed the purified amicyanin to be homogeneous.

Cytochrome c_H was partially purified from the eluate of the second hydroxyl apatite column. After addition of ammonium sulfate (90% saturation) to the eluate, the resulting solution was applied to a CM-32 cellulose column, then this eluate was applied to a Sephadex G-75 column. Cytochrome c_L absorbed on the top of the TEAE-cellulose column was eluted with a linear gradient of NaCl (0-0.4 M). Cytochrome c_L was partially purified by the chromatographies of the hydroxyl apatite and the Sephadex G-75 columns.

RESULTS

Molecular weight The molecular weight of amicyanin was estimated to be 10,800 and 12,000 by SDS polyacrylamide gel electrophoresis and Sephadex G-75 gel chromatography, respectively.

Amino acid composition The amino acid composition of amicyanin was found to be as follows: Asp (7), Thr (8), Ser (6), Glu (15), Pro (8), Gly (10), Ala (11), Cys (1), Val (13), Met (2), Ile (3), Leu (3), Tyr (2), Phe (4), Lys (10), His (3), Arg (2), and Trp (1). The values in parentheses are the nearest integer. The total number of the amino acids was 109. A molecular weight of 11,723 for amicyanin, which was calculated by the amino acid analysis, was used throughout the present work.

Copper content Atomic absorption spectroscopy indicated that 42 μg of the purified amicyanin contains 0.12 μg of copper atoms. This value corresponds to 0.55 atom of copper per mole of amicyanin, indicating that approximately half of the copper atom was liberated from amicyanin during purification.

Absorption spectrum The visible-ultraviolet absorption spectrum of the purified amicyanin is shown in Fig. 1. Absorption maxima were visible at 278, 460, 596, and 760 nm.

Apo-amicyanin Apo-amicyanin could be prepared by dialysing the purified amicyanin against 50 mM KCN (19), however, both the absorption peak at 596 nm and the activity as an electron acceptor were eliminated. When apo-amicyanin was titrated with a standard copper solution, its blue color and its activity were restored. After titration was completed, absorbance was 140% and activity was 86%, as compared with those of the purified amicyanin. The greater value for the absorbance can be explained by the partial liberation of copper during purification. The lesser value for the activity may have been caused by a partial denaturation of amicyanin and/or apo-amicyanin during dialysis. The millimolar extinction coefficient of amicyanin at 596 nm was calculated as 4.5 $mM^{-1}cm^{-1}$ from the titration of apo-amicyanin with the standard copper solution. This value was used to calculate the concentration of amicyanin.

Amicyanin as an electron carrier Amicyanin was found to be able to replace PMS in the PMS-DCPIP assay. This suggested that amicyanin could serve as a primary electron acceptor of methylamine dehydrogenase. The reduction rate of amicyanin at 596 nm was spectrophotometrically measured at 20°C. The

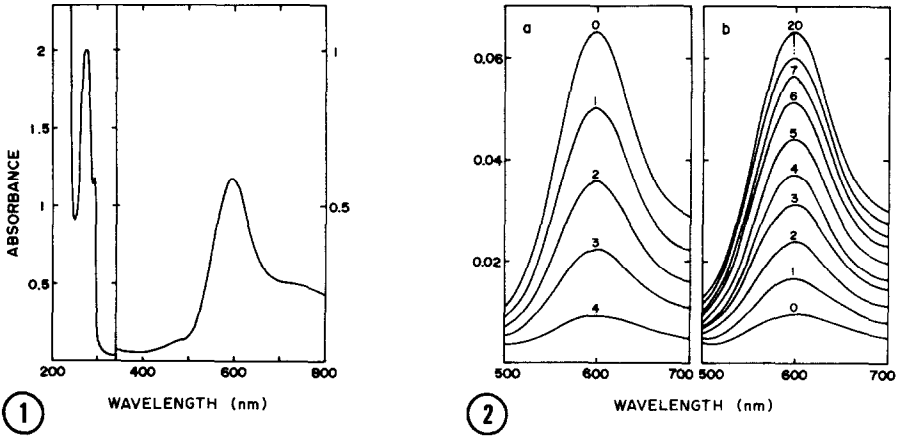

Fig. 1. Absorption spectrum of amicyanin. Cuvette contained 4.9 mg of the purified amicyanin in 2 ml of 10 mM K-phosphate buffer, pH 7.0.

Fig. 2. Titration of amicyanin with methylamine and ferricyanide. The reaction mixture containing 13.6 μM amicyanin and 0.15 μM methylamine dehydrogenase in 1.5 ml of 5 mM K-phosphate buffer, pH 7.0, was titrated in steps 0 to 4 with 5 μl of 5 mM methylamine hydrochloride, as in Fig. 2a. Subsequently, in steps 0 to 20, 5 μl of 0.53 mM ferricyanide was added to this solution, as in Fig. 2b.

reaction mixture contained 6.7 mM methylamine hydrochloride and 1.5 nM methylamine dehydrogenase in a total volume of 1 ml of 10 mM phosphate buffer, pH 7.0; and the concentration of amicyanin was changed from 3.7 μM to 9.3 μM. Using a double reciprocal plot, apparent K_m and V_{max} were calculated as 8.7 μM and 4900 μmoles amicyanin reduced per min per μmole of methylamine dehydrogenase, respectively. Under similar conditions, amicyanin was first titrated with methylamine in the presence of methylamine dehydrogenase (Fig. 2a), then this partially-reduced amicyanin was reversely titrated with ferricyanide (Fig. 2b). By stoichiometry, methylamine : amicyanin : ferricyanide was calculated as 1 : 1.9 : 2.1. This result indicates that amicyanin is a one-electron acceptor.

As cytochrome *c* was assumed to be the most probable natural electron acceptor of the reduced amicyanin, the reduction rate of cytochromes *c* at 550 nm was measured (Table I). Horse heart cytochrome *c*, the partially-purified *Pseudomonas* AM1 cytochromes c_H and c_L (20), and *Methylomonas* J cytochromes *c*-551 (I) and (II) were not appreciably reduced in the absence of amicyanin (5,10,13), but they were reduced in the presence of amicyanin. This result indicates that

Table I. Cytochrome *c* as electron acceptor of reduced amicyanin.

Cytochrome *c*	Final conc.(μM)	ΔA_{550}/min
Pseudomonas AM1 cyt c_H	5.1	0.040
Pseudomonas AM1 cyt c_L	11.0	0.036
Methylomonas J cyt *c*-551 (I)	7.9	0.048
Methylomonas J cyt *c*-551 (II)	7.0	0.040
Horse heart cyt *c*	6.2	0.011

Reaction mixture contained 6.7 mM methylamine hydrochloride, 0.6 μM amicyanin, and 3 nM methylamine dehydrogenase in 1 ml of 5 mM K-phosphate buffer, pH 7.0. Concentration of cytochromes *c* is indicated. The reduction rate of cytochromes *c* at 550 nm was spectrophotometrically recorded at 20°C.

a cytochrome *c* becomes an electron acceptor of reduced amicyanin and that specificity in the reoxidation reaction of reduced amicyanin as regards cytochrome *c* is broad.

DISCUSSION

Amicyanin was a type I blue copper protein with an ESR spectrum similar to that of the *Pseudomonas* blue protein called azurin. The properties of amicyanin, however, were different from those of the *Pseudomonas* blue protein, but resembled those of blue copper proteins purified from *Paracoccus denitrificans* (21) and *Alkaligenes faecalis* Strain-6 (22) in molecular weight and cystein content. In addition, our preliminary experiment on the N-terminal amino acid sequence of amicyanin showed that the amino acid sequence of the two blue copper proteins was different. Therefore, amicyanin might be classified as a new group of the type I blue copper protein. The oxidation-reduction potential (E_o') of amicyanin appeared to be low, possibly as low as that of stellacyanin (+180 mV), because the reduced amicyanin could be oxidized by DCPIP (+217 mV). The E_o' of the *Paracoccus* blue copper protein has been reported to be +230 mV (21).

The present work demonstrates that amicyanin serves as an electron carrier between methylamine dehydrogenase and cytochrome *c* *in vitro*. We propose that the electron transport chain of methylamine-grown *Pseudomonas* AM1 is:

$$CH_3NH_2 \xrightarrow{\text{methylamine dehydrogenase}} \text{amicyanin} \longrightarrow \text{cyt. } c \longrightarrow$$

It is interesting to note that amicyanin is located immediately in front of cytochrome *c*, in contrast to its position in both the respiratory and photosynthetic electron transport chains.

It can be assumed that amicyanin was induced in methylamine-grown *Pseudomonas* AM1, a facultative methylotroph, because no significant amount was found in the cell-free extract of methanol-grown cells, even though a blue copper protein, which had no amicyanin activity, was found. A blue copper protein, which could be reduced by methylamine dehydrogenase, was also found in the cell-free extract of methylamine-grown *Methylomonas* J, an obligate methylotroph. On the other hand, methanol-grown *Methylomonas* J contained a blue copper protein similar to the *Pseudomonas* blue protein which could scarcely be reduced by methylamine dehydrogenase but could be reduced by the reduced cytochromes *c*-551 (I) and (II) (13). We can therefore conclude that the above electron transport system operates in methylamine-grown facultative and obligate methylotrophs. Furthermore, the two kinds of functionally different blue copper proteins, amicyanin-type (low E_o') and azurin-type (high E_o'), are present in these methylotrophs and their amounts seem to be regulated by the growth carbon source.

REFERENCES

1. Eady, R.R. and Large, P.J. (1968) Biochem. J. 106, 245-255.
2. Eady, R.R. and Large, P.J. (1971) Biochem. J. 123, 757-771.
3. Shirai, S., Matsumoto, T., and Tobari, J. (1978) J. Biochem. 83, 1599-1607.
4. Mehta, R.J. (1977) Can. J. Microbiol. 23, 402-406.
5. Matsumoto, T. (1978) Biochim. Biophys. Acta 522, 291-302.
6. Matsumoto, T., Hiraoka, B.Y., and Tobari, J. (1978) Biochim. Biophys. Acta 522, 303-310.
7. Matsumoto, T. and Tobari, J. (1978) J. Biochem. 83, 1591-1597.
8. Matsumoto, T. and Tobari, J. (1978) J. Biochem. 84, 461-465.
9. Matsumoto, T., Shirai, S., Ishii, Y., and Tobari, J. (1980) J. Biochem. 88, 1097-1102.
10. Large, P.J., Meiberg, J.B.M., and Harder, W. (1979) FEMS Microbiol. Lett. 5, 281-286.
11. Boulton, C.A. and Large, P.J. (1979) Biochim. Biophys. Acta 570, 22-30.
12. De Beer, R., Duine, J.A., Frank, J., and Large, P.J. (1980) Biochim. Biophys. Acta 622, 370-374.
13. Ohta, S. and Tobari, J., J. Biochem. (in press).
14. Eady, R.R., Jarman, T.R., and Large, P.J. (1971) Biochem. J. 125, 449-459.
15. Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.

16. Andrews, P. (1964) Biochem. J. 91, 222-233.
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
18. Drabkin, D.R. (1942) J. Biol. Chem. 146, 605-617.
19. McMillin, D.R., Rosenberg, R.C., and Gray, H.B. (1974) Proc. Nat. Acad. Sci. U.S.A. 71, 4760-4762.
20. O'Keeffe, D.T. and Anthony, C. (1980) Biochem. J. 192, 411-419.
21. Martinkus, K., Kennelly, P.J., Pea, T., and Timkovich, R. (1980) Arch. Biochem. Biophys. 199, 465-472.
22. Kakutani, T., Watanabe, H., Arima, K., and Beppu, T. (1981) J. Biochem. 89, 463-472.