[44] Soluble Cytochromes c of Methanol-Utilizing Bacteria

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Introduction

Methanol-utilizing bacteria typically contain relatively large amounts of at least two soluble, periplasmic *c*-type cytochromes.[1,2] These are functionally distinct but spectrally almost indistinguishable; they were therefore named according to their isoelectric points. Cytochrome c_H has a higher isoelectric point (it is usually basic) and is now known to correspond to the typical soluble *c*-type cytochromes mediating oxidation by membrane oxidases in mitochondria and many bacteria. Cytochrome c_L occurs only in methylotrophs; it is always acidic (p*I* 3.0–5.0) and its mass (M_r 17,000–22,000) is about twice that of typical soluble *c*-type cytochromes. It is the specific electron acceptor for methanol dehydrogenase[1-3] and is sometimes induced to higher levels during growth on methanol, when it constitutes 30–70% of the total soluble *c*-type cytochrome.

Although these are the main soluble *c*-type cytochromes in methylotrophic bacteria, many bacteria contain others that appear very similar but which show slight differences of isoelectric point or molecular weight, and some methylotrophs also contain additional c-type cytochromes, some of which have unusual features but no known function.

It should be noted that multiple *c*-type cytochromes are often observed in membrane fractions having properties identical to those isolated from soluble fractions. It is also important to remember that ion-exchange chromatography is able to separate the oxidized and reduced forms from each other; it is sometimes convenient therefore to include 1 m*M* sodium ascorbate in buffers to keep all the cytochromes in the reduced state. Deamidation (conversion of amides to acids) of cytochromes may lead to the appearance of additional cytochromes, and in the case of at least one methylotroph *(Methylophilus methylotrophus)* a 4K fragment appears to be readily lost from cytochrome c_L, thus leading to the appearance of two forms of this cytochrome.

The purification methods given below are for the two main soluble *c*-type cytochromes of *Methylobacterium extorquens* AM1. These are likely

[1] C. Anthony, *Adv. Microb. Physiol.* **27,** 113 (1986).
[2] C. Anthony, *in* "Bacterial Energy Transduction" (C. Anthony, ed.), p. 293. Academic Press, London, 1988.
[3] M. Beardmore-Gray, D. T. O'Keeffe, and C. Anthony, *J. Gen. Microbiol.* **132,** 1553 (1983).

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to be readily adaptable to most other organisms, but for convenience references to alternative purification methods for the following important methylotrophs are given: *M. extorquens*,[4] *Methylophilus methylotrophus*,[3,5,6] *Acetobacter methanolicus*,[7] *Hyphomicrobium* X,[8] *Paracoccus denitrificans*,[9-11] *Methylomonas* J,[12] and organism 4025.[13]

Assay of *c*-Type Cytochromes

The assay to be used depends on the purpose and the state of purification of the cytochromes. The assay depends on the characteristic absorption spectra of the cytochromes. These are so similar that different *c*-type cytochromes cannot be distinguished in crude extracts and a measure of total cytochrome *c* must be used. The main components are usually separated early on in any procedure, either by ion-exchange chromatography or by gel filtration, and the proportions of each cytochrome can then be determined.

Units. The amount of cytochrome is recorded in micromoles, nanomoles, etc. This is calculated from the visible absorption spectrum of the reduced form at the absorption maximum (about 550 nm). For a rough estimate during purification the extinction coefficient for the reduced form may be used. In crude extracts it should be noted that the baseline in the visible region is likely to be sloping, and it is often convenient to use an artificial base of a line between about 535 and 575 nm for calculating peak heights. For more accurate estimates the reduced minus oxidized difference spectrum is used, with the appropriate extinction coefficient. A few grains of dithionite is used to reduce cytochromes, and a few grains of potassium ferricyanide or ammonium persulfate is used for oxidation. When either the proportions of the various cytochromes in crude extracts or the extinction coefficients are not known, a reasonable estimate of the amount of cytochromes can be made by assuming extinction coefficients from the literature for other cytochromes. Useful average values are 29 mM^{-1} cm^{-1} for cytochrome c_H, and 25 mM^{-1} cm^{-1} for cytochrome

[4] D. T. O'Keeffe and C. Anthony, *Biochem. J.* **192,** 411 (1980).
[5] A. R. Cross and C. Anthony, *Biochem. J.* **192,** 421 (1980).
[6] S. J. Froud and C. Anthony, *J. Gen. Microbiol.* **130,** 3319 (1984).
[7] E. J. Elliott and C. Anthony, *J. Gen. Microbiol.* **134,** 369 (1988).
[8] M. Dijkstra, J. Frank, and J. A. Duine, *Biochem. J.* **257,** 87 (1989).
[9] M. Husain and V. L. Davidson, *J. Biol. Chem.* **261,** 8577 (1986).
[10] K. A. Gray, D. B. Knaff, M. Husain, and V. L. Davidson, *FEBS Lett.* **207,** 239 (1986).
[11] G. Bosma, M. Braster, A. H. Stouthamer, and H. W. van Verseveld, *Eur. J. Biochem.* **165,** 665 (1987).
[12] S. Ohta and J. Tobari, *J. Biochem. (Tokyo)* **90,** 215 (1981).
[13] S. A. Lawton and C. Anthony, *Biochem. J.* **228,** 719 (1985).

c_L. For a pure cytochrome the extinction coefficient is calculated from the measured molecular weight and the heme content as determined from the pyridine hemochrome spectrum.

Molecular Weights by Denaturing Electrophoresis. Although not strictly an assay for cytochrome *c*, an important additional analysis is the determination of molecular weights by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). This is important for confirming the presence of the expected cytochrome in fractions. Electrophoresis is done at pH 8.3 using SDS–Tris–glycine with final acrylamide concentrations of 10–15%, and gels are stained with Coomassie Brilliant Blue as previously described;[14] suitable molecular weights standards are used [BDH 44262 2I (Poole, UK), Sigma Dalton Mark VII (St. Louis, MO), and Pharmacia PMW kits (Piscataway, NJ)]. To confirm the position of cytochromes, they are visualized prior to protein staining by their activity in the peroxidase assay; if this is to be used, mercaptoethanol is not included in the electrophoresis system.

Reagents for Peroxidase Assay

TMBZ (3,3′,5,5′-tetramethylbenzidine), 136 mg in 90 ml methanol (prepare fresh)
Sodium acetate buffer, 0.25 *M* (pH 5.0) (14.3 ml glacial acetic acid plus 900 ml water, adjusted to pH 5.5 with 1 *M* NaOH and made up to 1 liter)
TMBZ–acetate, 90 ml TMBZ solution plus 210 ml sodium acetate buffer
Hydrogen peroxide, 30% (w/v)
2-Propanol–acetate buffer, 3 parts 2-propanol plus 7 parts acetate buffer

Procedure. The gel is immersed in acetate buffer for 15 min to fix the protein; it is then immersed in the TMBZ–acetate mixture for 30–60 min in the dark. Hydrogen peroxide is added (1 ml/300 ml of solution). The green-staining cytochromes become visible within 15 min, and staining intensifies over 45 min. After sufficient staining is observed the gel is immersed in 2-propanol–acetate buffer mixture to remove excess stain and to clear the gel; the mixture can be replaced a few times to improve clarity. The gels can be photographed or scanned at this stage prior to staining for total protein in Coomassie blue. It is recommended that a range of protein concentrations be used for each fraction to ensure observation of minor components.

[14] K. Weber and M. Osborn, "The Proteins," 3rd ed. (H. Neurath and R. L. Hill, eds.). Vol. 1, p. 179. Academic Press, London, 1975.

Purification of Cytochromes from *Methylobacterium extorquens* AM1

Growth of *Methylobacterium extorquens* AM1 and preparation of extracts are described in the chapter on the methanol dehydrogenase of this organism.[15]

Cytochrome c_L. The soluble fraction from about 15 g wet weight of bacteria is passed down a column (24 × 130 mm) of DEAE-Sepharose (Pharmacia, Fast Flow) equilibrated in 20 m*M* Tris-HCl (pH 8.0). Methanol dehydrogenase and cytochrome c_H are not adsorbed; cytochrome c_L is eluted (at about 100 m*M* NaCl) with a gradient of 0–250 m*M* NaCl (total, 200 ml) in the same buffer. Without dialysis, solid ammonium sulfate is added to give 40% saturation at 4°, and the precipitate formed after centrifugation for 10 min at 10,000 *g* is discarded. The supernatant is applied to a Pharmacia Phenyl-Superose column (1 ml) equilibrated in 20 m*M* MOPS containing 2.0 *M* ammonium sulfate (pH 7.0) and cytochrome eluted (at about 1.6 *M* ammonium sulfate) with a decreasing gradient of 2–1 *M* ammonium sulfate (total volume about 20 ml). The cytochrome is desalted on a PD10 column equilibrated in 20 m*M* Tris-HCl (pH 8.0) and applied to a Pharmacia Mono Q column equilibrated in the same buffer. Pure cytochrome c_L is eluted (at about 100 m*M*) with a gradient of 0–200 m*M* NaCl in the same buffer (total volume about 10 ml). It is desalted on a PD10 column and stored at −20° (See Table I for purification steps.)

Cytochrome c_H. After passage through DEAE-Sepharose (see above) the extract is dialyzed overnight at 4° against 20 liters of 10 m*M* potassium phosphate buffer (pH 7.0) and applied to a hydroxylapatite column (16 × 100 mm) equilibrated in the same phosphate buffer. This is eluted with a linear gradient of 10–250 m*M* potassium phosphate (pH 7.0), the cytochrome *c* being eluted just prior to the dehydrogenase (at about 80 m*M* phosphate). It is dialyzed against 20 m*M* sodium acetate buffer (pH 5.0), applied to a column (24 × 85 mm) of S-Sepharose (Pharmacia, Fast Flow) equilibrated with 25 m*M* MES buffer (pH 5.5), and eluted (at about 90 m*M* NaCl) with a linear gradient of NaCl in the same buffer (0–250 m*M*, 200 ml total volume). For smaller amounts S-Sepharose can be replaced by a Mono S column, equilibrated in 25 m*M* MES buffer (pH 5.5), and the cytochrome c_H eluted as a very sharp peak using a linear gradient of 0–150 m*M* NaCl in the same buffer (total volume, 15 ml). The pure protein is passed through PD10 and stored at −20°.

[15] D. J. Day and C. Anthony, this volume [33].

TABLE I
PURIFICATION OF SOLUBLE CYTOCHROMES FROM *Methylobacterium extorquens* AM1[a]

Purification stage	Volume (ml)	Total cytochrome (nmol)	Specific content (nmol/mg)	Purification (-fold)	Yield (%)
Crude extract	75	1200	1.63	1	100
Cytochrome c_H					
DEAE-Sepharose	110	406	1.84	1.1	34
Hydroxylapatite	19	348	10.0	6.5	29
S-Sepharose	4.3	240	160.0	98	20
Cytochrome c_L					
DEAE-Sepharose	19	298	19.6	12.0	25
Phenyl-Superose	11	240	39.5	24.2	20
Mono Q	8	216	45.0	27.6	18

[a] The cytochrome recorded in the crude extract is total *c*-type cytochrome. The percentage values for each separate cytochrome relate to the initial total cytochrome content.

Properties of Cytochrome c_L of *Methylobacterium extorquens* AM1[4,16–18]

Cytochrome c_L has a molecular weight of 18,735 (from the gene sequence), a p*I* of 4.2, a midpoint redox potential at pH 7.0 of +256 mV (the apparent standard midpoint potential at pH 0 is +345 mV). Absorption maxima in the reduced form are at 416 and 549 nm, with extinction coefficients of 163 and 26 mM^{-1} cm^{-1}. In the oxidized form the absorption maxima are at 410 and 695 nm, with an extinction coefficient at 695 nm of 0.35 mM^{-1} cm^{-1}. The extinction coefficient at 549 nm measured from the reduced minus oxidized difference spectrum is 21.8 mM^{-1} cm^{-1}.

Cytochrome c_L has a single low-spin heme prosthetic group which has histidine and methionine axial ligands. It reacts with CO slowly and incompletely (72% binding in buffer saturated with CO at room temperature). It is rapidly autoreducible at high pH and it reacts with methanol dehydrogenase. Four ionizations affect the redox potential between pH 4.0 and 9.5, two in the oxidized form (p*K* values about 3.6 and 5.6) and two in the reduced form (p*K* values about 4.4 and 6.4), suggesting that the ionizing groups involved may be the two propionate side chains of the heme. The amino acid sequence (derived from the gene sequence) shows that cytochrome c_L constitutes a novel class of *c*-type cytochromes.

[16] M. Beardmore-Gray, D. T. O'Keefe, and C. Anthony, *Biochem. J.* **207,** 161 (1982).
[17] D. T. O'Keefe and C. Anthony, *Biochem. J.* **190,** 481 (1980).
[18] D. N. Nunn and C. Anthony, *Biochem. J.* **256,** 673 (1988).

Properties of Cytochrome c_H *Methylobacterium extorquens* AM1[4,16,17]

Cytochrome c_H has a molecular weight of about 11,000, a p*I* of 8.8, and a midpoint redox potential at pH 7.0 of +294 mV (the apparent standard midpoint potential at pH 0 is +404 mV). Absorption maxima in the reduced form are at 416.5 and 550.5 nm, with extinction coefficients of 162 and 31 mM^{-1} cm^{-1}. In the oxidized form the absorption maxima are at 410 and 695 nm, with an extinction coefficient at 695 nm of 0.5 mM^{-1} cm^{-1}. The extinction coefficient at 550 nm measured from the reduced minus oxidized difference spectrum is 22.5 mM^{-1} cm^{-1}.

Cytochrome c_H has a single low-spin heme prosthetic group which has histidine and methionine axial ligands. It reacts with CO slowly and incompletely (36% binding in buffer saturated with CO at room temperature). It is rapidly autoreducible at high pH but does not react with methanol dehydrogenase. Four ionizations affect the redox potential between pH 4.0 and 9.5, two in the oxidized form (p*K* values about 3.5 and 5.5) and two in the reduced form (p*K* values about 4.5 and 6.5), suggesting that the ionizing groups involved may be the two propionate side chains of the heme. The amino acid sequence shows that cytochrome c_H is a typical class 1 *c*-type cytochrome.

[45] Cytochrome c_L and Cytochrome c_H from *Hyphomicrobium* X

By J. FRANK and J. A. DUINE

All gram-negative methylotrophic bacteria investigated so far contain at least two soluble cytochromes *c*, indicated as cytochrome c_L and cytochrome c_H, the subscripts referring to a low and a high isoelectric point, respectively.[1-3] It is generally accepted that cytochrome c_L is the electron acceptor of methanol dehydrogenase.[4,5] Different views exist, however, on the mechanism involved in this electron-transfer process,[5,6] the discrepancy being related to the autoreduction behavior of the isolated cytochrome c_L. As reported already, the procedure developed for the isola-

[1] D. T. O'Keeffe and C. Anthony, *Biochem. J.* **190,** 481 (1980).
[2] D. T. O'Keeffe and C. Anthony, *Biochem. J.* **192,** 411 (1980).
[3] M. Dijkstra, J. Frank, J. E. van Wielink, and J. A. Duine, *Biochem. J.* **251,** 467 (1988).
[4] C. Anthony, *Adv. Microb. Physiol.* **27,** 162 (1986).
[5] M. Dijkstra, J. Frank, and J. A. Duine, *Biochem. J.* **257,** 87 (1989).
[6] M. Beardmore-Gray, D. T. O'Keeffe, and C. Anthony, *J. Gen. Microbiol.* **115,** 523 (1983).

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