The organisation of methanol dehydrogenase and *c*-type cytochromes on the respiratory membrane of *Methylophilus methylotrophus*

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The membrane-impermeant, protein-labelling reagent [^{14}C]isethionyl acetimidate has been used to investigate the detailed topography of the membrane-associated methanol oxidase system in the methylotrophic bacterium *Methylophilus methylotrophus*. The results show that methanol dehydrogenase and cytochrome c_1 are significantly less exposed on the periplasmic surface of the membrane than either cytochrome c_H or an as yet unidentified 12-kDa protein. The partial crypticity of the methanol dehydrogenase has been confirmed by fingerprinting with V8 protease. The results are discussed in terms of the electron transfer functions of the various redox proteins.

Respiratory chain *Methylophilus methylotrophus* *Respiratory membrane*

1. INTRODUCTION

The methylotrophic bacterium *Methylophilus methylotrophus* oxidises methanol via an energy-conserving methanol oxidase system that is comprised, according to the exact conditions of growth, of a PQQ-linked methanol dehydrogenase, cytochromes c_H and c_L, and cytochrome oxidase aa_3 or *o* [1–8].

Previous analyses in this laboratory of periplasm and cytoplasm fractions prepared from whole cells of *M. methylotrophus* have shown that methanol dehydrogenase and the *c*-type cytochromes are restricted to the periplasm fraction [9,10]. In addition, the effect of EDTA and related chelating agents on methanol oxidation by whole cells of *M. methylotrophus* has indicated that methanol dehydrogenase, cytochrome c_H and approx. one-third of the cytochrome c_L are loosely attached to the periplasmic side of the membrane, probably via the mediation of Mg^{2+} [11,12]; the remainder of the cytochrome c_L, and the cytochrome oxidases, are firmly membrane bound. The presence of methanol dehydrogenase and the *c*-type cytochromes on the periplasmic side of the membrane has recently been elegantly confirmed by labelling with [^{14}C]IEA [13].

This paper describes various labelling experiments using [^{14}C]IEA which show that several major proteins including methanol dehydrogenase, the two *c*-type cytochromes and a 12-kDa protein are quite differently exposed on the periplasmic surface of the respiratory membrane. The results are discussed in terms of the spatial organisation and electron transfer functions of the methanol oxidase system.

Abbreviations: IEA, isethionyl acetimidate; Mdh, methanol dehydrogenase

2. MATERIALS AND METHODS

2.1. *Preparation of whole cells*

M. methylotrophus (NC1B 10515) was grown in methanol-limited continuous culture (D = 0.18 h^{-1}; 40°C) [3]. After harvesting, cells were washed once in 20 mM sodium phosphate buffer, pH 8.0, and then resuspended in the same buffer

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to a cell density of 15 mg/ml. Periplasm and cytoplasm fractions were prepared from cells resuspended in 20 mM Tris–HCl, pH 7.5, plus 0.75 M mannitol as in [9].

2.2. *Radiochemical labelling with IEA*

[1-^{14}C]IEA was obtained from Amersham International (Amersham) at a specific activity of 58.1 mCi/mmol. Since the compound is extremely susceptible to hydrolysis, the ampoule was opened in a completely dry atmosphere and subdivided into approximately equal, smaller aliquots which were then stored at −15°C in sealed tubes surrounded by drying agent. Labelling with [^{14}C]IEA was carried out using a modification of the method described in [13]. An aliquot of [^{14}C]IEA was added to a freshly-prepared stock solution of unlabelled IEA in 20 mM phosphate buffer, pH 8.0, then immediately mixed with a suspension of whole cells (final concentrations 10 mM IEA and 10 mg cells/ml) and incubated at room temperature for 30 min. The cells were then pelleted by centrifugation, and washed twice in 20 mM phosphate buffer, pH 8.0. The labelled cell suspension (10 mg cells/ml) was sonicated for 4 × 30 s periods and then subjected to differential centrifugation (15000 × *g* for 15 min, followed by 100000 × *g* for 1 h) to yield a clear orange-pink supernatant (SI).

Alternatively, cells were pre-exposed to unlabelled IEA (10 mM), then washed once in 20 mM phosphate buffer, pH 8.0, sonicated as above and exposed to [^{14}C]IEA (10 mM) for 30 min at room temperature; a supernatant fraction (SII) was subsequently prepared as described above.

2.3. *SDS–polyacrylamide gel electrophoresis*

Discontinuous SDS–PAGE was carried out in a vertical slab-gel system (LKB, Bromma, Sweden) using resolving gels which contained 12.5% acrylamide and 3.75% bisacrylamide [14]. The gels were stained for protein with Kenacid blue R (BDH, Poole) and destained by normal procedures. Haemoproteins were detected by staining with 3,3′,5,5′-tetramethylbenzidine [15]. Excised methanol dehydrogenase bands were fingerprinted at room temperature using the method of [16]; 4 μg of V8 protease from *Staphylococcus aureus* was present in each track, and electrophoresis was stopped for 1 h while the dehydrogenase was in the stacking gel to allow digestion to take place.

2.4. *Analysis of labelled bands*

Gels were prepared for fluorography by soaking for 30 min in Amplify (Amersham), then drying at 60°C for 2 h under vacuum. The dried gels were stored in contact with preflashed Fuji RX X-ray film at −70°C for up to 21 days. Alternatively, selected bands were excised from the stained gels and solubilised by immersing in 0.5 ml NCS solubiliser for 2 h at 50°C; 4.5 ml of toluene-based scintillation fluid Fisofluor 3 (Fisons, Loughborough, England) was added to each sample and the radioactivity was determined in a liquid scintillation counter [17].

3. RESULTS

Periplasm and cytoplasm fractions prepared from whole cells of *M. methylotrophus* exhibited strikingly different protein patterns following SDS–polyacrylamide gel electrophoresis (PAGE) (fig.1), even after allowing for the presence in the periplasm fraction of the RNase, DNase and lysozyme which had been added during the sphaeroplast preparation procedure. Several distinctive marker proteins could be identified in each fraction including those of 58 kDa (methanol dehydrogenase subunit), 28 kDa, 18 kDa (cytochrome c_L), 12 kDa and 10–11 kDa (cytochrome c_H) in the periplasm, and of 64, 46 and 24 kDa in the cytoplasm.

SDS–PAGE of supernatant fractions SI (prepared from whole cells labelled with [^{14}C]IEA), and SII (prepared from whole cells exposed to unlabelled IEA, then sonicated and labelled with [^{14}C]IEA) yielded gels with almost identical protein patterns (fig.2), which included all of the periplasm and cytoplasm markers identified above. The two supernatants also exhibited similar haem-staining patterns, viz., a strong band at 18 kDa (cytochrome c_L) and a weaker band at 11 kDa (cytochrome c_H). In contrast, autofluorography of supernatants SI and SII revealed quite different radiochemical labelling patterns. Thus, supernatant SI exhibited relatively few radioactive bands, but these included three strong bands with molecular masses of 58 kDa (methanol dehydrogenase), 12 and 11 kDa (cytochrome c_H), and 3 weaker bands with values of 85, 28 and 18 kDa (cytochrome c_L), i.e., all 5 of the periplasm markers plus an 85-kDa protein

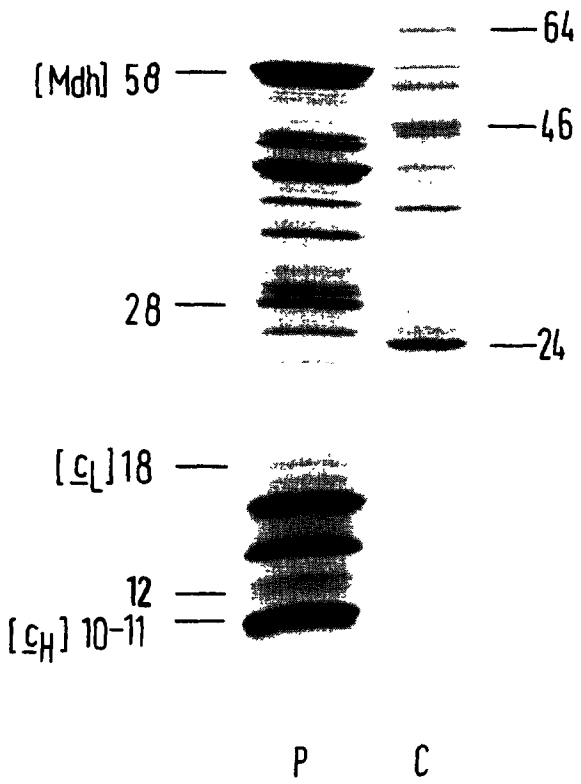

Fig.1. SDS–PAGE of periplasm and cytoplasm fractions prepared from whole cells of *M. methylotrophus*. P, periplasm (from 0.3 mg cells); C, cytoplasm (from 0.6 mg cells). The gel was stained for protein. Haem staining of parallel tracks (not shown) confirmed that the proteins of M_r 18000 and approx. 10500 were cytochromes, probably c_L and c_H, respectively.

which is presumably released from the membrane by sonication but not by treatment with lysozyme–EDTA. Importantly, no significant labelling of any of the cytoplasm markers was observed, thus confirming the previous report [13] that [^{14}C]IEA penetrates the outer membrane, but not the cytoplasmic membrane, of methylotrophic bacteria. Supernatant SII exhibited a substantial number of radioactive bands, including all 6 bands labelled in supernatant SI, plus the 3 cytoplasm markers (64, 46 and 24 kDa).

Quantitative analysis of the radiochemical labelling patterns shown above confirmed that identical bands excised from SDS–PAGE gels of supernatants SI and SII had bound widely varying amounts of [^{14}C]IEA (table 1). The cytoplasm markers exhibited fairly constant, high ratios (SII/SI) of bound [^{14}C]IEA, thus confirming the evidence from the autofluorograms that the cytoplasmic membrane was indeed impermeable to this reagent. In contrast, the 5 periplasm markers and the 85-kDa protein exhibited lower and much more variable ratios, indicating that these proteins, although present on the periplasmic side of the membrane, do not occupy uniformly exposed positions. Since a high ratio is indicative of high crypticity, it must be concluded that these proteins are exposed in the order 85 kDa < 28 kDa < cytochrome c_L < methanol dehydrogenase < cytochrome c_H < 12 kDa. The 85-kDa protein is thus particularly well-hidden, as indeed might be expected from the previous observation that sonication is required to release it from the membrane, whereas at the other end of the range the 12-kDa protein is highly exposed. The latter can by no means be regarded as being a soluble periplasmic protein, however, since as such it would exhibit a labelling ratio approaching zero, i.e., it would be completely radiochemically labelled in supernatant SI but remain unlabelled in supernatant SII.

The apparent partial crypticity of methanol dehydrogenase in whole cells was further investigated by fingerprinting the radiochemically-labelled enzyme following its excision from SDS–PAGE gels of supernatants SI and SII. Exposure of the excised enzyme to *S. aureus* V8 protease during SDS–PAGE yielded at least 8 major proteolysis products with M_r values ranging from 31000 to 13000, compared with an M_r of 58000 for the non-proteolysed enzyme. The average ratio (SII/SI), from 5 separate experiments, of the amount of [^{14}C]IEA bound to identical proteolysis products was very close to that observed with the parent methanol dehydrogenase (i.e., 11.3, cf., 10.4). Strikingly, however, the range of values was very much wider (i.e., 4.3–20.8, cf., 9.5–12.0) thus supporting the view that [^{14}C]IEA labels different regions of the methanol dehydrogenase molecule depending on whether the molecule is attached to the membrane or whether it has been pre-

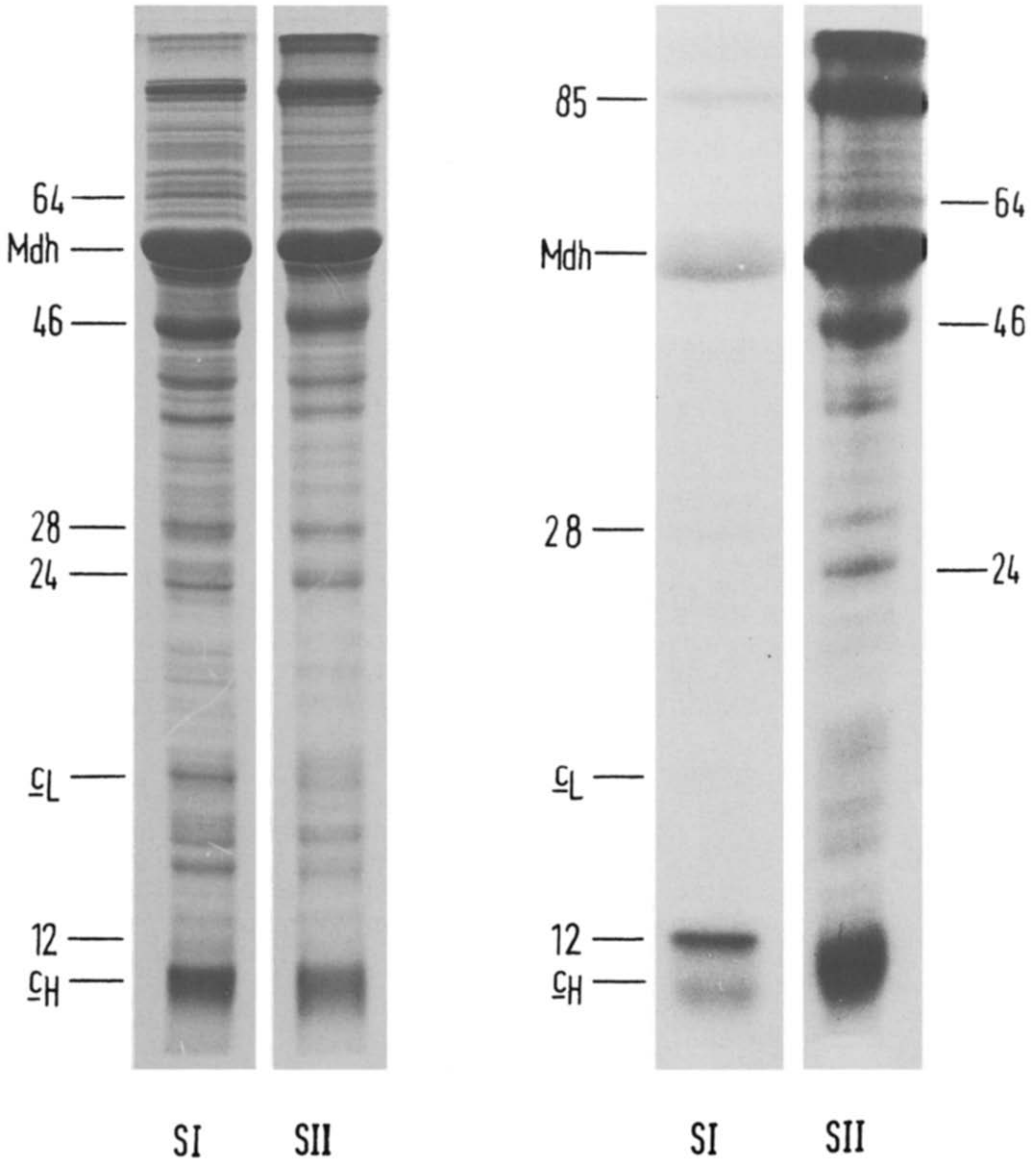

Fig.2. SDS–PAGE of supernatants SI and SII prepared from whole cells of *M. methylotrophus*. Each track contained 205 μg protein. Following electrophoresis, the gel was cut into strips which were then stained for protein (left-hand tracks) or were dried and subjected to autofluorography (right-hand tracks).

exposed to unlabelled IEA and then released from the membrane by sonication.

It follows from this that maximum labelling by [^{14}C]IEA of methanol dehydrogenase, or indeed of any of the other loosely membrane-bound proteins, will only occur when it has been released from the membrane (e.g., by sonication or exposure to EDTA) without having previously been exposed to unlabelled IEA. This was confirmed by comparing the total amount of [^{14}C]IEA bound by methanol dehydrogenase, the *c*-type cytochromes and the 12-kDa protein in the two supernatant fractions (SI + SII) with the amount bound by the same proteins following exposure of whole cells to EDTA (10 mg cells/ml; 1 mM EDTA); similar values were obtained in each case.

Table 1

The labelling of selected proteins of *M. methylotrophus* with [^{14}C]IEA

Molecular mass of protein (kDa)	[^{14}C]IEA ratio (SII:SI)
64	59
46	54
24	48
85	27
58 (Mdh subunit)	10
28	16
18 (cytochrome c_L)	13
12	2
11 (cytochrome c_H)	7

Radiochemically labelled supernatants SI and SII were prepared from *M. methylotrophus*, then subjected to SDS–PAGE and stained for protein. After destaining, pairs of protein bands were excised from the gels, solubilised in NCS and assayed for radioactivity. The labelling ratios quoted for each protein are the average of at least 4 separate determinations

4. DISCUSSION

The results described in this paper confirm that the amino group labelling reagent [^{14}C]IEA does not penetrate the cytoplasmic membrane of *M. methylotrophus* [13], and also show that methanol dehydrogenase, cytochrome c_H and some of the cytochrome c_L (together with unidentified 85-, 28- and 12-kDa proteins) are loosely attached to the periplasmic side of the respiratory membrane in such a way that they exhibit quite different labelling ratios (SII/SI). Thus methanol dehydrogenase and cytochrome c_L are significantly less accessible to labelling than is cytochrome c_H.

Whole cells of *M. methylotrophus* grown under methanol-limited conditions contain a cytochrome c_H:methanol dehydrogenase:easily released cytochrome c_L ratio of approx. 1:1:0.3 [11,12]. The concentrations of these components are high, and indeed have been calculated to be sufficient easily to cover the periplasmic surface of the membrane [6]. In view of this it seems likely that the intact methanol dehydrogenase (M_r 116000, i.e., composed of two 58000 subunits) is loosely attached to the membrane such that it is largely surrounded by cytochrome c_L overlaid with cytochrome c_H. This relatively well-hidden locus of loosely membrane-bound cytochrome c_L is compatible with the view that it accepts electrons from methanol dehydrogenase and transfers them either to cytochrome c_H, or under some conditions, to the cytochrome $c_L o$ complex which is embedded in the membrane [6,8]. Similarly, the more highly exposed position of cytochrome c_H is commensurate with its likely role of transferring electrons to cytochrome oxidase aa_3 which, by analogy with higher organisms, might be expected to protrude significantly from the periplasmic side of the membrane [18]. The entire methanol dehydrogenase-cytochromes *c* complex is probably stabilised, and loosely anchored to the periplasmic side of the membrane, by various electrostatic forces including cross-bridging via Mg^{2+} [11,12].

Neither the relatively well-hidden 85- and 28-kDa proteins, nor the highly exposed 12-kDa protein, have yet been identified. It should be noted, however, that a blue copper protein (amicyanin, M_r 12000) is present in cell extracts of *Pseudomonas* AM1 and *Methylomonas* J grown on methylamine, and an azurin-type copper protein has also been detected in these organisms following growth on methanol [19]. Furthermore, small copper proteins of the azurin or rusticyanin type are usually found in the periplasm or loosely attached to the periplasmic side of the membrane. It is possible, therefore, that the 12-kDa protein present in *M. methylotrophus* is a copper protein with an as yet unidentified role in methanol oxidation.

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REFERENCES

[1] Cross, A.R. and Anthony, C. (1980) Biochem. J. 192, 421–427.
[2] Cross, A.R. and Anthony, C. (1980) Biochem. J. 192, 429–439.

[3] Dawson, M.J. and Jones, C.W. (1981) Biochem. J. 194, 915–924.
[4] Dawson, M.J. and Jones, C.W. (1981) Eur. J. Biochem. 118, 113–118.
[5] Beardmore-Gray, M., O'Keefe, D.T. and Anthony, C. (1982) Biochem. J. 207, 161–165.
[6] Beardmore-Gray, M., O'Keefe, D.T. and Anthony, C. (1983) J. Gen. Microbiol. 129, 923–933.
[7] Dawson, M.J. and Jones, C.W. (1982) Arch. Microbiol. 133, 55–61.
[8] Carver, M.A. and Jones, C.W. (1983) FEBS Lett. 155, 187–191.
[9] Jones, C.W., Kingsbury, S.A. and Dawson, M.J. (1982) FEMS Microbiol. Lett. 13, 195–200.
[10] Burton, S.M., Byrom, D., Carver, M.A., Jones, G.D.D. and Jones, C.W. (1983) FEMS Microbiol. Lett. 17, 185–190.
[11] Carver, M.A., Humphrey, K.M., Patchett, R.A. and Jones, C.W. (1984) Eur. J. Biochem. 138, 611–615.
[12] Carver, M.A. and Jones, C.W. (1984) Arch. Microbiol., in press.
[13] Kasprzak, A.A. and Steenkamp, D.J. (1983) J. Bacteriol. 156, 348–353.
[14] Laemmli, U.K. (1970) Nature 277, 680.
[15] Thomas, P.E., Ryan, D. and Leven, W. (1976) Anal. Biochem. 75, 168–176.
[16] Cleveland, D.W., Fischer, S.G., Kirschner, M.W. and Laemmli, U.K. (1977) J. Biol. Chem. 252, 1102–1106.
[17] Basch, R.S. (1968) Anal. Biochem. 26, 184–188.
[18] Capaldi, R.A., Malatesta, F. and Darley-Usmar, V.M. (1983) Biochim. Biophys. Acta 726, 135–148.
[19] Tobari, J. and Harada, Y. (1981) Biochem. Biophys. Res. Commun. 101, 502–508.