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CLONING AND SEQUENCING OF THE STRUCTURAL GENE FOR THE SMALL SUBUNIT OF METHYLAMINE DEHYDROGENASE FROM *METHYLOBACTERIUM EXTORQUENS* AM1: EVIDENCE FOR TWO TRYPTOPHAN RESIDUES INVOLVED IN THE ACTIVE CENTER

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SUMMARY: In two independent clone libraries, clones were identified that hybridized with oligonucleotide probes based on N- or C-terminal polypeptide sequence of the small subunit of methylamine dehydrogenase from *Methylobacterium extorquens* AM1. Plasmids from all clones had in common a 5.2 kb *Bam*HI-*Hin*dIII DNA fragment. A 0.57 kb *Sac*II-*Bcl*I subfragment that hybridized to the oligonucleotide probes was sequenced. Nucleotide sequence analysis coincided with polypeptide sequence data in the structural part of the small subunit with a single contradiction: amino acid 17 is Asp rather than Asn. The two amino acids that are involved in the active center which had not been determined from previous polypeptide sequencing proved to be tryptophans.

Methylobacterium extorquens AM1 can use methylamine as a sole source of carbon and energy and oxidizes this compound via methylamine dehydrogenase (MADH; EC 1.4.99.4; 1). The enzyme consists of two large (MW 40,000 d) and two small subunits (MW 13,000 d; 2) with a covalently bound cofactor whose structure is not yet resolved. Ishii *et al.* (3) sequenced the small subunit polypeptide and presented evidence that the cofactor of MADH is linked to two unidentified amino acid residues. Amino acid sequence has also been proposed for the MADH small subunit from *Thiobacillus versutus* based on X-ray crystallographic analysis (4), and this sequence has 77% similarity to that from *M. extorquens* AM1. Two unidentified amino acids were proposed to be linked to the cofactor, and these were in a similar

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Abbreviations: MADH, methylamine dehydrogenase; PQQ, pyrroloquinoline quinone; kb, kilobase; d, daltons.

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position to the two unidentified residues in the polypeptide from *M. extorquens* AM1.

The identity of the MADH cofactor is still not clear. Mass spectral data suggested that the cofactor of MADH from the restricted obligate methylotroph Bacterium W3A1 (now defined as a *Methylophilus sp.*; 5) is a pyrroloquinoline quinone (PQQ) derivative (6). Van der Meer *et al.* (7) proposed that the cofactor of MADH from *T. versutus* was PQQ, but later it was suggested that MADH in this bacterium contained not PQQ but rather a PQQ-like cofactor (pro-PQQ, 4). However, the proposed structure fit the electron density maps imperfectly (4). Covalently bound PQQ or PQQ-like cofactors have been suggested for several other enzymes, including bovine serum amine oxidase (8), bovine adrenal dopamine hydroxylase (9), human placental lysyl oxidase (10) and methylamine oxidase from the Gram-positive methylotrophic *Arthrobacter* species (11), but these are also now uncertain. Robertson *et al.* (12) suggested that the bovine adrenal dopamine hydroxylase does not contain PQQ, and Janes *et al.* (13) have obtained data that the active site of bovine serum amine oxidase contains 6-hydroxydopa (TOPA) but not PQQ or its derivatives. These data raise the possibility that the cofactor in MADH may not be a PQQ derivative. In order to investigate the nature of the MADH cofactor, it is necessary to identify the two amino acid residues that are involved in the active center. Therefore, the gene for the small subunit of MADH from *M. extorquens* AM1 was identified, cloned and sequenced.

MATERIALS AND METHODS

Strains and plasmids. Wild type *M. extorquens* AM1 (14) was used for chromosomal DNA preparations. All plasmid constructions and sequencing were done with plasmids of the pUC series (Pharmacia; Piscataway, NJ) or plasmid pTZ19TT (15) in *Escherichia coli* strain DH5α (F^-, *rec*A1, Δ*lac*ZYA, $r^- m^-$, *thi*-1, *gyr*A66, *sup*E44, *end*A1, φ80*lac*Z M15).

DNA manipulations. Plasmid isolation, *E.coli* strain transformation, sucrose gradient fractionation of *M. extorquens* AM1 chromosomal DNA, restriction, ligation and polynucleotide kinase reactions, blot and colony hybridization were carried out as described by Maniatis *et al.* (16).

Clone libraries. A clone library constructed in the vector pVK100 was used (17). A partial clone library was also constructed by ligating to pUC7 a fraction of *Bam*HI-generated fragments of *M. extorquens* AM1 chromosomal DNA isolated from a sucrose gradient.

Oligonucleotide probe synthesis. Two oligonucleotide probes were synthesized based on known amino acid sequence of the small subunit polypeptide (Fig 1; Caltech Microchemical Facility). One probe was synthesized on the basis of the N-terminal polypeptide sequence (N-probe) with 32-fold redundancy. A second probe was synthesized based on the C-terminal polypeptide sequence (C-probe) with a redundancy of 64 fold.

DNA sequencing. DNA sequencing was performed by the dideoxy chain termination method (18) according to instructions of the manufacturer (Sequenase, US Biochemical Corp., Cleveland, OH).

RESULTS AND DISCUSSION

Two oligonucleotide probes were generated based on N-terminal and C-terminal amino acid sequence of the MADH small subunit (Fig. 1). The specificity

(a) GAA\G GAT\C GAT\C GCG\C ATG ACG\C TAT\C CAT\C TG

Glu Asp Asp Ala Met Thr Tyr His Cys 64

GAA GAC GAT GCG ATG ACC TAC CAC TG

(b) AAI TGG AAI CCG\C CAA\G GAT\C AAT\C AAT\C GT

Lys Trp Lys Pro Gln Asp Asn Asn Val 32

AAG TGG AAG CCG CAG GAC ACC GAC GT

Figure 1. Sequences of (a) C-probe and (b) N-probe for the MADH small subunit. The first line in either case is the nucleotide sequence of the probe, the second line is the corresponding amino acid sequence (3) and degree of redundancy, and the third line is the determined nucleotide sequence. The amino acids are for (a) positions 111-119 and for (b) positions 10-18.

of each oligonucleotide probe was verified by hybridization with *Bam*HI, *Sma*I and *Eco*RI digests of *M. extorquens* AM1 chromosomal DNA. At a hybridization and wash temperature of 50°C, both probes hybridized only with one chromosomal DNA fragment in any digest pattern, and in each case both probes hybridized to the same fragment (data not shown). The C-probe gave a considerably stronger hybridization signal than the N-probe. The smallest fragment of chromosomal DNA that hybridized with either probe was a 7.3 kb *Bam*HI fragment.

A *Bam*HI digest of chromosomal DNA was fractionated on a sucrose gradient, and fragments in the range of 6.0 kb to 8.5 kb were used for constructing a partial clone library in pUC7. 200 clones from the *Bam*HI partial clone library and an additional 1400 clones from a *Hin*dIII partial-digest clone library constructed in the vector pVK100 (17) were hybridized with the C-probe. One clone from the *Bam*HI library and five clones from the *Hin*dIII library were identified. The clone from the *Bam*HI library contained a plasmid with a 7.3 kb *Bam*HI insertion in pUC7 (pAYC142), which hybridized with both the C-probe and N-probe. Four of five clones from the *Hin*dIII library contained a 9.3 kb *Bam*HI fragment which hybridized with both the C-probe and N-probe. The fifth contained a 13.4 kb *Bam*HI fragment which also hybridized with both probes. The 9.3 kb and 13.4 kb fragments were subcloned into pUC7, resulting in the plasmids designated pAYC144 and pAYC146x, respectively. pAYC142, pAYC144 and pAYC 146x contained a 5.2 kb *Bam*HI-*Hin*dIII fragment that had similar restriction maps in each (Fig. 2) and hybridized with both the C-probe and N-probe. This fragment was subcloned into pTZ19TT (plasmid pAYC147).

The small subunit polypeptide gene was localized within the 5.2 kb *Bam*HI-*Hin*dIII fragment by hybridization of different restriction digests of pAYC147 with C- and N-probes. The C-probe hybridized with the small *Bam*HI-*Pvu*II fragment shown in Fig. 2 and the N-probe hybridized with the adjacent *Pvu*II-*Xho*I fragment. Both fragments were subcloned and sequenced and the probe sites identified (Fig. 3). Figure 4 shows the DNA sequence encoding the structural portion of the mature

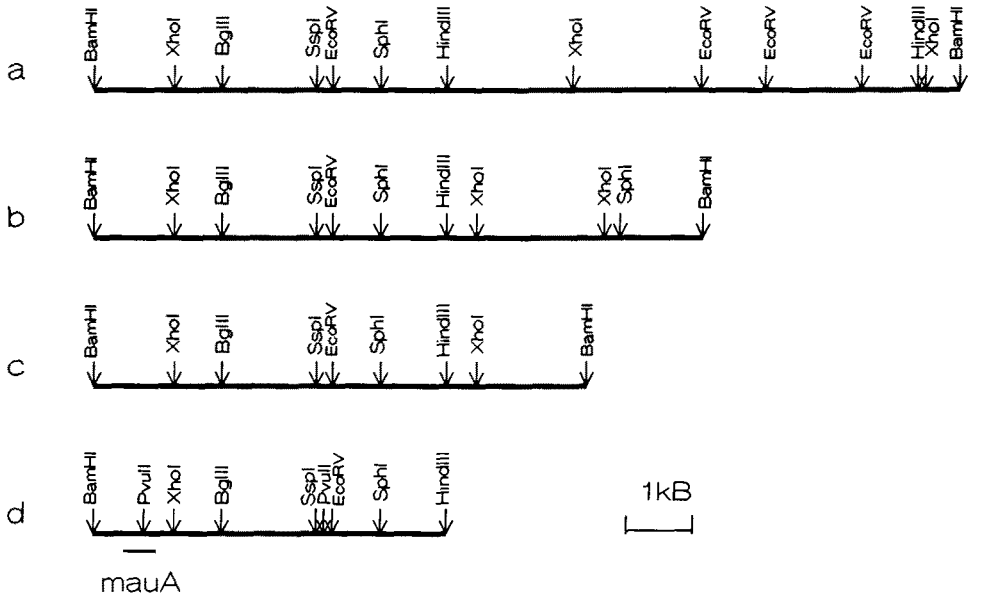

Figure 2. Restriction maps of *M. extorquens* AM1 DNA fragments in plasmids (a) pAYC146x, (b) pAYC144, (c) pAYC142 and (d) pAYC147. The small subunit gene (*mau*A) location is noted in (d).

MADH small subunit polypeptide and the predicted amino acid sequence. We propose to designate this gene *mau*A, for *m*ethyl*a*mine *u*tilization.

The mature polypeptide of the MADH small subunit has a calculated molecular weight of 13,949 d, which is in agreement with SDS-PAGE determinations (2). Frequencies of codon usage in this sequence show strong preference for G or C in the third position, as observed in sequences of other genes from *M. extorquens* AM1 (19). A search of GenBank and EMBL DNA and protein sequence data bases revealed no significant similarity of the MADH coding region to other sequences.

The published protein sequence data coincide with that derived from the DNA sequence with a sole exception: amino acid 17 is Asp rather than Asn. Since

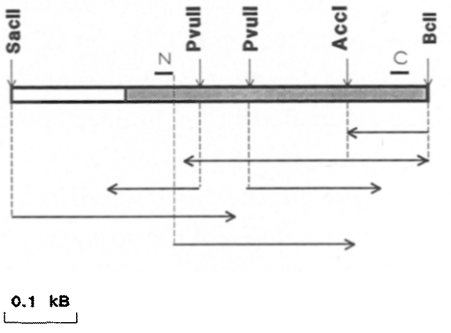

Figure 3. Map of the DNA fragment containing the gene for the small subunit of MADH (filled segment) and the sequencing strategy. Lines above the box represent sites of N-probe (N) and C-probe (C) binding.

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  1 GCC GAG AGC GCG GGC GAT CCA CGC GGT AAG TGG AAG CCG CAG
  1 Ala Glu Ser Ala Gly Asp Pro Arg Gly Lys Trp Lys Pro Gln

 43 GAC AAC GAC GTC CAG TCC TGC GAT TAC TGG CGT CAC TGC TCC
 15 Asp Asn Asp Val Gln Ser Cys Asp Tyr Trp Arg His Cys Ser

 85 ATC GAC GGC AAC ATC TGC GAC TGC TCC GGC GGC TCG CTC ACC
 29 Ile Asp Gly Asn Ile Cys Asp Cys Ser Gly Gly Ser Leu Thr

127 AGC TGT CCG CCT GGC ACC AAG CTC GCC TCC AGC TCC TGG GTT
 43 Ser Cys Pro Pro Gly Thr Lys Leu Ala Ser Ser Ser Trp Val

169 GCC AGC TGC TAC AAC CCA ACT GAC AAG CAG AGC TAC CTG ATC
 57 Ala Ser Cys Tyr Asn Pro Thr Asp Lys Gln Ser Tyr Leu Ile

211 TCC TAC CGC GAT TGC TGC GGC GCC AAC GTG TCC GGC CGC TGT
 71 Ser Tyr Arg Asp Cys Cys Gly Ala Asn Val Ser Gly Arg Cys

253 GCC TGC CTG AAC ACC GAA GGT GAG CTG CCG GTC TAC CGT CCG
 85 Ala Cys Leu Asn Thr Glu Gly Glu Leu Pro Val Tyr Arg Pro

295 GAG TTC GGT AAC GAC ATC ATC TGG TGC TTC GGC GCC GAA GAC
 99 Glu Phe Gly Asn Asp Ile Ile Trp Cys Phe Gly Ala Glu Asp

337 GAC GCG ATG ACC TAC CAC TGC ACG ATC TCG CCG ATC GTC GGC
113 Asp Ala Met Thr Tyr His Cys Thr Ile Ser Pro Ile Val Gly

379 AAG GCG AGC TGA
127 Lys Ala Ser Ter
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Figure 4. Sequence of the gene encoding the mature MADH small subunit from *M. extorquens* AM1. The numbers refer to the nucleotide (upper) and the amino acid sequences (lower), respectively. The underlined amino acid is the Asp derived from nucleotide sequence that contradicts the polypeptide sequence data (3). Double underlined amino acids are tryptophans involved in the active center of the enzyme.

the N-probe was synthesized on the basis of Asn in position 17, it contained an AAC/T triplet instead of the correct GAC/T. This may explain the weaker binding of the N-probe in comparison with the C-probe. It is likely that the difference in sequences is due to an error in the original amino acid determination, since the sequence proposed for the MADH small subunit from *T. versutus* based on X-ray crystallographic analysis (4) also places Asp in the equivalent position. However, it is possible that the difference reflects a single substitution mutation that could exist in two separated lines of *M. extorquens* AM1 cultivated in different laboratories. One ambiguity present in the *M. extorquens* AM1 polypeptide sequence was at position 39, where both Lys and Leu were detected (3), but the DNA sequence suggested this residue was Lys.

The two amino acids involved in the active site that were not identified by polypeptide sequencing are tryptophans. Given the constraints of the known active site geometry (4) and the comparatively low reactivity of tryptophans it seems unlikely that tryptophan residues would serve as simple connectors for a cofactor in the active site. It has been proposed that the cofactor 6-hydroxydopa could be generated from an active site tyrosine of bovine serum amine oxidase by post-translational modification (13). Since these two tryptophans of MADH are known to be covalently attached at the site of the cofactor (3, 4, 6) it is possible that they are post-translationally modified and connected to form a novel reactive center in MADH .

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