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A new model for the pro-PQQ cofactor of quinoprotein methylamine dehydrogenase

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A model for the pro-PQQ cofactor of *Thiobacillus versutus* methylamine dehydrogenase was fitted into a 2.25 Å resolution electron density distribution for this enzyme. This proposed model of pro-PQQ consists of a tyrosine-derived quinone indole bicyclic structure. Linkage of the cofactor to the light subunit of the enzyme occurs via the side chain of glutamate 57, which is itself bound to the side chain of arginine 107 of that subunit. Since the elements of this cofactor are derived from tyrosine, glutamate and arginine, we have named it 'TGA pro-PQQ'.

Methylamine dehydrogenase; Pyrroloquinoline quinone, pro-; Crystallographic model building; (*Thiobacillus versutus*)

1. INTRODUCTION

The Gram negative bacterium *Thiobacillus versutus* is a methylotroph, i.e. it is capable of growth using C1 compounds as sole source of carbon and energy. When methylamine is the growth substrate, the first step in the carbon dissimilation pathway is the oxidation of the substrate by the enzyme methylamine dehydrogenase (MADH) (EC 1.4.99.3) to obtain formaldehyde and ammonia [1]. The reducing equivalents are then transferred to a c-type cytochrome via a type I blue copper protein, amicyanin [2].

MADH is a tetrameric enzyme, made up of two heavy (H) and tow light (L) subunits [3]. In the early stage of the characterization of this enzyme, it became obvious that it contained a very tightly bound organic cofactor, which resembled that of the copper-containing diamine oxidase enzymes (EC 1.4.3.6) [4]. Later, ESR and ENDOR spectra of the native enzyme indicated that the cofactor had a quinone structure, and that it was very similar to the cofactor found in the enzyme methanol dehydrogenase (MDH) (EC 1.1.99.8) [5]. Since the latter was identified as pyrroloquinoline quinone (or PQQ) [6,7], the MADH cofactor was also assumed to be PQQ, or a closely related compound. The observed differences in the ENDOR spectra of MDH and MADH were attributed either to differences in the structures of the two cofactors, or to differences in the interactions between cofactor and enzyme [5].

Sequence studies carried out on the L subunit of the *Pseudomonas AM1* MADH revealed that the enzyme cofactor was covalently bound to two residues of that subunit [8], but the nature of the residues binding the cofactor could not be established. More recently, mass spectroscopy of the semicarbazide adduct of the cofactor of the MADH from *Bacterium W3A1* suggested that this prosthetic group had a tricyclic PQQ nucleus-like structure, but lacking the three carboxylate groups of PQQ [9]. Linking of the cofactor to the L subunit of MADH was proposed to occur via one Cys thioether bond and one Ser oxygen ether linkage. However, derivatization of the intact, protein bound cofactor of the *T. versutus* enzyme with phenylhydrazine (PH), followed by proteolysis and

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acid treatment gave compounds with the same spectroscopic and chromatographic properties as the adducts of authentic PQQ with PH [10]. Therefore, it was concluded that the MADH cofactor was integral PQQ, linked to the L subunit via pronase-sensitive peptide or ester bonds.

In order to elucidate the active site of this enzyme with its unusual cofactor, as well as to study the exact role of this PQQ-related cofactor in the enzyme-catalyzed reaction and subsequent electron transfer, we have determined the 3D structure of the MADH from *Thiobacillus versutus* by X-ray crystallographic methods. A 2.25 Å resolution electron density map for the native enzyme was recently obtained, in which the polypeptide chain could be followed in both the H and the L subunits [11]. In this map, the electron density corresponding to the quinone cofactor suggested that only two of the three rings of PQQ were present in the MADH cofactor. Since PQQ biosynthesis is thought to involve the addition of a glutamate residue to a tyrosine-derived quinone indole bicyclic ring structure [12,13], we proposed the presence in MADH of a similar, 'open-form' pro-PQQ cofactor having a 9a-γ-glutamyl indole structure [11].

However, while this previous model of the pro-PQQ cofactor fitted nicely into the electron density of the cofactor region, we experienced problems when trying to model the postulated linkages from the prosthetic group both to the side chain of residue 57, and to that of residue 107 of the L subunit. In order to shed more light on the exact nature of the cofactor of MADH, we have been carrying out further model building studies in the electron density obtained for this cofactor. The molecular structure for the cofactor presented in this paper agrees with the available chemical information.

2. EXPERIMENTAL

A 2.25 Å resolution density distribution for *T. versutus* MADH was calculated from structure factor amplitudes obtained from crystals grown as described previously [14]. Phases were initially determined by the isomorphous replacement method, complemented in later stages by solvent flattening and crystallographic refinement of the atomic model [11]. The model used for phase calculation consisted of the polypeptide chains of both subunits of the enzyme, in which an X-ray sequence had been assigned to each subunit type [11]. This model gave an *R*-factor value of 28.6% with all measured data from 6.0 to 2.25 Å resolution.

Graphic work was carried out on an Evans & Sutherland PS390 picture system, where electron density maps and molecular models were displayed simultaneously. This, together with the interactive manipulation of models and regularization of these models using Jan Hermans algorithm [15] was carried out with the macromolecular crystallography display and model building program FRODO [16]. The quinone indole model was docked manually into the native electron density corresponding to the cofactor region in the active site of MADH. Using the SAM option of the program FRODO [16], the side chains of the two cofactor binding residues were mutated from alanine to glutamine and from serine to arginine at positions 57 and 107 of our previously determined X-ray sequence [11] of the L subunit. These two side chains were positioned optimally into the remaining (i.e. not occupied by the quinone indole moiety) electron density at the cofactor site by rotation about single bonds, followed by regularization of the resulting structure.

3. RESULTS AND DISCUSSION

When the native 2.25 Å resolution electron density distribution of *T. versutus* MADH, became available, we were able to fit a complete model for the polypeptide chains of both H and L subunits into this density [11]. During this step, assignment of side chains had been done solely on the basis of the electron density, and of the interactions which were observed between these side chains and other parts of the structure. The establishment of this 'X-ray sequence' was necessary due to the lack of any sequence information available at that time.

A partial preliminary amino acid sequence has recently been obtained for a short stretch of 31 residues in the H subunit of the *T. versutus* enzyme (Huitema, F., Beintema, J.J. and Duine, J.A., personal communication). The quality of the electron density map which was used to deduce the X-ray sequence can be judged from the results of the comparison of this X-ray sequence with the chemical sequence: in this short segment we observed 14 residues which had been perfectly identified from the electron density map while the remaining 17 residues differed marginally from the correct chemical sequence. In particular the solvent inaccessible residues agreed well in the two sequences. Therefore, we believe that a reasonable amount of structural information can be deduced from our map.

The electron density corresponding to the quinone cofactor of MADH indicates that only the quinone indole portion of the PQQ molecule can

be found in the cofactor of this enzyme. The remaining density of this cofactor region is not coplanar with the density corresponding to the quinone indole group, and the 9a-γ-glutamyl indole moiety which we proposed earlier as the pro-PQQ cofactor of MADH gives a nice fit into the electron density [11].

However, some difficulties were encountered for the proposed linkages of this cofactor to the enzyme: there is not enough density to accomodate without strain the proposed C9′ ester bond to residue 57, while the distance between the N6 atom of the cofactor and the side chain atom of residue 107 is too long for the proposed linkage. Therefore, a major revision of the pro-PQQ model was required to better explain the electron density.

In doing so the following information has been taken into account: (i) glutamate and tyrosine are the two precursors of PQQ biosynthesis [12,13]; (ii) the procedure used for the extraction of PQQ

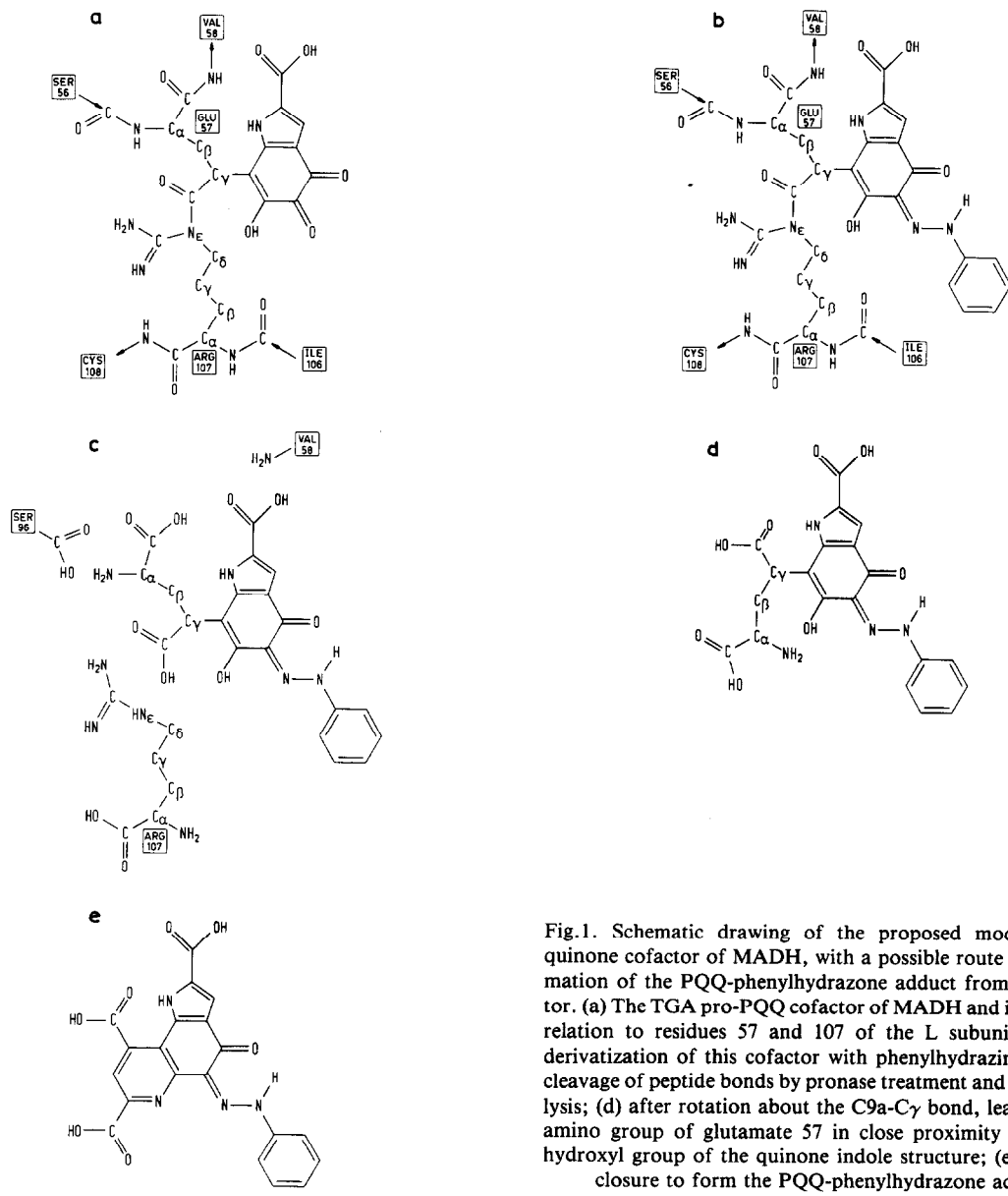

Fig.1. Schematic drawing of the proposed model for the quinone cofactor of MADH, with a possible route for the formation of the PQQ-phenylhydrazone adduct from this cofactor. (a) The TGA pro-PQQ cofactor of MADH and its proposed relation to residues 57 and 107 of the L subunit; (b) after derivatization of this cofactor with phenylhydrazine; (c) after cleavage of peptide bonds by pronase treatment and acid hydrolysis; (d) after rotation about the C9a-Cγ bond, leaving the α-amino group of glutamate 57 in close proximity to the C5a hydroxyl group of the quinone indole structure; (e) after ring closure to form the PQQ-phenylhydrazone adduct.

from MADH occurs in conditions in which peptide bonds can be hydrolyzed or proteolytically cleaved [10]; (iii) during chemical synthesis of PQQ, ring closure from an 'open-form' bicyclic ring intermediate takes place easily to generate the tricyclic PQQ nucleus structure [17]. This resulted in an improved proposal for the structure of the pro-PQQ cofactor of MADH (fig.1a). This cofactor contains the previously described quinone indole moiety [11], which is bound by a $C9_a$-Cγ linkage to the side chain of Glu-57. The latter is in turn linked via a peptide bond involving its γ-carboxylate group to the Nϵ atom of Arg-107. Since the elements of this quinone cofactor are derived from three amino acids, namely tyrosine, glutamate and arginine, we propose that it should be named 'TGA pro-PQQ'.

The generation of the PQQ-phenylhydrazone adduct from MADH [10] can be easily explained from this model (fig.1): after formation of the hydrazone at the C5 position of the quinone indole group, pronase treatment and acid hydrolysis will produce the 9a-γ-glutamyl indole hydrazone, which undergoes cyclization and oxidation in the reaction conditions [10] to yield the required product.

The current model for the MADH cofactor, including the postulated linkages to the side chains of residue 57 and 107 of the L subunit, gives a nice fit into the electron density, whose features are now all explained by this model (fig.2). The proposed orientation of the quinone indole fused ring structure has been confirmed by the calculation of a 3.0 Å resolution $|F_o - F_c|$ difference Fourier map using amplitudes obtained from a crystal which had been treated with a phenylhydrazine [11], and phases from that partially refined structure with an R factor of 24.2% for data between 8.0 and 3.0 Å resolution. This map showed, in addition to the electron density for the MADH cofactor, that corresponding to the inhibitor, connected at the proposed C5 position of the quinone indole group.

In view of the proposed in situ synthesis of the cofactor, both for those quinoproteins containing non-covalently bound PQQ [13,18], as well as for those containing a covalently bound cofactor [19], the current model for the pro-PQQ cofactor of MADH is an attractive hypothesis: both synthesis and linkage of this cofactor to MADH might proceed on the enzyme itself. With respect to the 'covalent' class of quinoproteins, it is interesting to consider the enzyme diamine oxidase, whose cofactor bears resemblance to that of MADH [4]. Recently, results were reported of the sequence analysis of a tryptic peptide containing the phenylhydrazone adduct of the cofactor, obtained from the pig kidney enzyme [20]. These results led to the

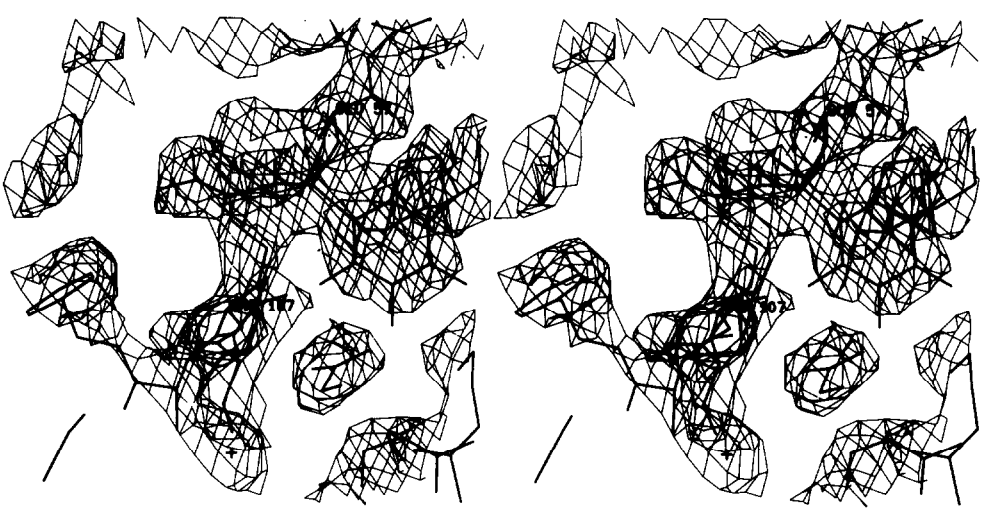

Fig.2. Stereo view of the active site of MADH showing the proposed TGA pro-PQQ structure and the protein model in this region of the molecule (thick lines), together with the corresponding 2.25 Å resolution electron density contoured at 1σ above the average of the map (thin lines). This electron density map was calculated using combined solvent flattening and refined protein model phases. The atoms of the cofactor were not included for crystallographic refinement and phase calculation in order to minimize model bias. Labels indicate the Cα positions of cofactor binding residues 57 and 107.

proposal of this cofactor being a PQQ molecule, linked to the protein via a peptide bond between the C2 carboxylate group of the cofactor and the Nζ atom of a lysine residue [20]. The model proposed in the current paper for the pro-PQQ cofactor of MADH also contains such a peptide bond, linking the Cγ (or C9 in the notation for the PQQ cofactor) carboxylate group of the quinone indole bound Glu-57 to the Nϵ atom of Arg-107. Since the reactions catalyzed by these enzymes differ only at the level of the transfer of electrons from the substrate to the respective electron acceptors, and since their respective cofactors are known to be similar [4], the diamine oxidase enzymes might also contain such an 'open-form' pro-PQQ cofactor.

The discovery that the electron density for the MADH cofactor does not accomodate the full ring system of PQQ indicates that the commonly used procedures to classify an enzyme as a member of the group of quinoproteins [5,10,21] are unable to distinguish between the different but related cofactors which are found in these proteins. Obviously, new procedures are required for the detection and characterization of PQQ and of PQQ related cofactors as they occur in the active sites of quinoproteins. Once such procedures are available, it will be most interesting to see how well our proposed TGA pro-PQQ cofactor will agree with the new experimental data.

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