Volume 333, number 1,2, 188–192 FEBS 13162 October 1993

N-terminal heterogeneity of methylamine dehydrogenase from *Thiobacillus versutus*

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Received 15 July 1993; revised version received 20 August 1993

The N-terminal processing of MADH from the bacterium *T. versutus* and the N-terminal heterogeneity of the isolated α subunit of the $\alpha_2\beta_2$ protein complex was demonstrated by a combination of Edman sequence analysis of an electroblotted band, in situ digested with pyroglutamate aminopeptidase, and accurate mass determination of the homogenous subunit by the technique of electrospray ionisation mass spectrometry. From this study, it appears that the corresponding gene of the α subunit contains 395 amino acids and that it is preceded by a leader sequence of 31 residues.

Methylamine dehydrogenase; α Subunit; N-terminal heterogeneity; Electrospray mass spectrometric analysis

1. INTRODUCTION

The oxidation of methylamine to formaldehyde and ammonia is a rather specific reaction catalyzed by the enzyme methylamine dehydrogenase (EC 1.4.99.3). As far as known, the reaction occurs in a limited number of bacterial species, such as e.g. *Thiobacillus versutus* [1], *Paracoccus denitrificans* [2] and *Bacterium* W3A1 [3]. MADH from *Thiobacillus versutus* is composed of 2 α and 2 β subunits. The molecular weight of each of the subunits has been determined by SDS-polyacrylamide gel electrophoresis to be respectively 49 and 14.1 kDa [1] and by gel filtration chromatography to be 47.5 and 13.9 kDa, respectively [4].

The tertiary structure of *Thiobacillus versutus* MADH is now known at a resolution of 2.25 Å [5]. The primary structure of the smaller β subunit has already been determined after cloning of the corresponding gene [6]. The cloning and sequencing of the gene coding for the α subunit has recently been finished [7]. The latter results allow the interpretation of the data described in the present paper which concerns the N-terminal sequencing and the precise nature of the heterogeneity of the methylamine dehydrogenase α subunit.

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Abbreviations: MADH, methylamine dehydrogenase; Pe-ase, pyroglutamate aminopeptidase; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; EDTA, ethylenediamine-tetraacetic acid; PVP, polyvinylpyrrolidone.

2. MATERIALS AND METHODS

2.1. *Electrophoresis and blotting*

Electrophoresis was carried out in a home-made apparatus on a 16.5% T/3% C gel of 70 × 70 × 1.2 mm, according to Schägger and von Jagow [8]. The proteins were electroblotted onto a Problott membrane (Applied Biosystems, USA) in a BioRad Transblot Cell (BioRad, USA) for 18 h, using 50 mM Tris-borate as a blotting buffer. After blotting, the bands were stained in a strongly diluted solution of Amido black, until they became clearly visible. The blot was allowed to dry in the air.

2.2. *Sequence and mass analysis*

Amino acid sequence analyses were performed on a 477A pulsed liquid sequencer, equiped with an on-line 120A PTH-analyzer (Applied Biosystems, USA). The specifically designed Blott Cartridge (Applied Biosystems, USA) was used for sequence analysis of the electroblotted proteins.

Electrospray mass spectrometry was carried out on a BIO-Q triple quadrupole mass spectrometer equiped with an electrospray ionisation source (Fisons VG, Altrincham, UK). Ten microliters of a sample solution in 50% methanol/1% acetic acid in water were injected manually in the 10 μl loop of the 5717 Rheodyne injector. Scans were registered during 2 min at a flow rate of 4 μl/min of 50% methanol/1% acetic acid in water delivered by a 140A Solvent Delivery System (Applied Biosystems, USA).

2.3. *In situ treatment of the electroblotted α subunit with pyroglutamate aminopeptidase*

After SDS-polyacrylamide gel electrophoresis of the α subunit, prepared as in [6], the excised band of the electroblotted components was cut into two parts and treated at room temperature with 100 μl of 0.2% PVP-40 (Sigma, USA) for 20 min. To this solution, 100 μl of water was added and incubated for 30 min at 30°C. After incubation, the excess PVP-40 was removed by extensive washing with two volumes of 200 μl water without vortexing. The blots were immersed in 70 μl of 0.1 M phosphate buffer (pH 7.8), 10 mM disodium EDTA, 5 mM EDTA and 5% glycerol. Pyroglutamate aminopeptidase treatment was carried out with an enzyme to substrate ratio of 1/15 for 12 h at

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room temperature and for 24 h at 4°C. The wet blot was dried under argon in the Blott Cartridge of the sequencer during 2 min.

2.4. *Treatment of the native protein with pyroglutamate aminopeptidase*

About 3.7 nmol of a solution of native MADH was desalted by ultrafiltration through a Centricon-30 membrane (Amicon, USA), using three volumes of water. Five out of the 50 μl of the purified and concentrated sample was used for mass analysis; the remaining volume was dried, prior to deblocking of the N-terminus with pyroglutamate aminopeptidase.

The dried purified protein was redissolved in 30 μl of buffer containing 0.1 M phosphate (pH 7.8), 10 mM disodium EDTA, 5 mM EDTA and 5% glycerol. Pyroglutamate aminopeptidase (Boehringer Mannheim, Germany) was added to this mixture, resulting in an enzyme to substrate ratio of 1/20 and incubated overnight at room temperature. Seven μl of this sample was applied on the sequencer and the rest was used for further purification by SDS-PAGE and electroblotting.

3. RESULTS AND DISCUSSION

3.1. *The heterogeneity of the α subunit*

Since the sequence of the β subunit was already available both from the gene and from peptides covering most of this sequence, the α subunit was first separated from the β subunit following the procedure described before [6]. Whereas the β subunit is stable when isolated separately, the α subunit, on the contrary, appears to undergo a particular and reproducible degradation pattern which can be made visible by SDS-PAGE (Fig. 1(I)). N-Terminal sequence analysis of the α subunit reveals a mixture of at least 4 residues at each position (result not shown) with the additional feature that the sum of the amounts of the phenylthiohydantoin derivatives is only about one fourth of the on average 30% initial yield that is normally detected by Edman degradation of a non-blocked protein.

After electroblotting of the α subunit fragments, we submitted them separately to N-terminal sequence analysis and obtained the quantitative results given in Table I. Band a, of the lowest mobility and an apparent molecular weight of around 43 kDa appeared to reveal no sequence information at all. Band b, covering the molecular weight range between 40 and 36 kDa did yield sequence information but appeared to contain two polypeptides of which the fourth residue was an alanine in each of them. Band c, which from its width compared to most of the reference proteins could be expected to be a pure fragment, also appeared to be a mixture of two polypeptides both starting with an arginine residue.

At that momemt, it became clear from the X-ray data

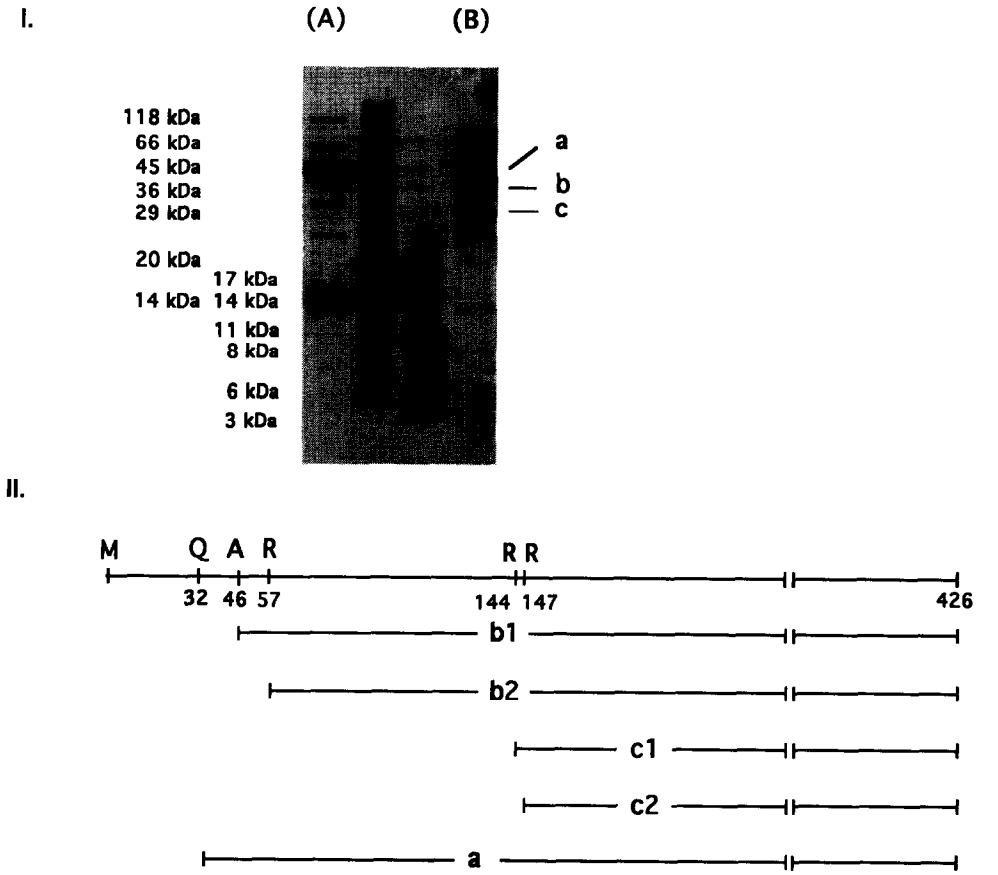

Fig. 1 (I) SDS-polyacrylamide gel electrophoresis of the native methylamine dehydrogenase (A) and of a preparation of the α subunit of MADH from *T. versutus* (B). The two central lanes represent the molecular weights reference proteins. (II) Schematic presentation of the gene sequence of the α subunit with indication of the start positions of the polypeptides that have been sequenced from the electroblotted bands b and c. The polypeptide chains terminate with the sequence Met-Asp-Ser (426). The polypeptide chain of the X-ray analysed protein covers the region 57–426.

available [9], that the sequence of fragment c2 could be identified in the backbone pattern of MADH. With difference of only three additional residues at the N-terminus of fragment c2, the sequence of c1 was the same as that of the 32 kDa fragment that had been found earlier and that has been used to proof the backbone tracing of the polypeptide chain in the X-ray structure [9]. From that latter analysis it had appeared that the 'crystallographic' N-terminus of the α subunit was 90 residues upstream the 32 kDa fragment sequence. The sequence of the blotted fragment b2 could now also be traced in the X-ray sequence. It suggested that this fragment started very near the N-terminal part of the 'X-ray sequence'. Clearly, the sequence of the α subunit gene was needed to resolve this problem as well as to determine whether all the other sequence data obtained from the electroblotted fragments were derived only from the α subunit and not from a contaminant protein.

When the gene sequence became available, the results of which will be published in detail separately [7], it became clear that not only the sequence Arg-Thr-Asp-Tyr-Val was part of the α subunit (band c2) but also the sequence of the other components of band c (c1: Arg-Gly-Lys-Arg-Thr) as well as the sequences of band b (b1: Ala-Ala-Asp-Ala-Ala, and b2: Arg-Gly-Ala-Ala-Glu). Fig. 1(II) gives a schematic presentation of the gene of the α subunit and indicates at which position the different polypeptides start. However, the problem remained of the exact position of the N-terminal residue of the α subunit and, in view of the crystallographic evidence for the location of peptide c2, also of the MADH in total.

3.2. *Mass analysis of the α subunit*

The molecular mass of the protein, calculated from the gene sequence is 46387.9 Da which is 5484 Da more than the molecular mass calculated if the N-terminal of peptide b2 would be the N-terminus of the α subunit. This difference suggested that the initial part of the gene might contain a leader sequence of around 3 kDa.

A very rapid way to determine the exact length of a protein, without having to follow the time-consuming way of sequencing all of the constituting peptides of a particular digest, consists in measuring the mass of the polypeptide by a mass analysis method of high accuracy. Such a technique, known as electrospray ionisation mass spectrometry has recently become available [10]. Its accuracy is generally accepted, from the experiments carried out on pure samples, to be around 0.01%. For a protein of 43,309.3 Da, this would be within the range of 4.33 Da. We carried out such an analysis on the α subunit, but as expected, obtained a complex spectrum from which it was difficult to identify the different components a, b1, b2, c1 and c2. We therefore repeated the mass analysis on a fresh sample of the α subunit

Table I

Sequence results, in pmol, for the three electroblotted bands of the α-subunit of methylamine dehydrogenase from *T. versutus*

Step	Band a	Band b				Band c				Band a (Pe-ase treated)	
		b2		b1		c1		c2			
1		Arg	19.1	Ala	21.3			Arg	20.7	Thr	29.2
2						Thr	18.2	Gly	18.4	Glu	9.9
3		Ala	17.9	Asp	2.9	Asp	7.5	Lys	6.1	Pro	16.9
4	Blocked		Ala 30.5			Tyr	13.2	Arg	3.7	Ala	13.5
5		Glu	26.7			Val	10.6	Thr	4.1	Glu	21.6
6		Ala	20.3	Gly	11.5	Glu	7.3	Asp	4.4	Pro	9.9
7				Gln	5.0	Val	4.6	Tyr	6.2	Glu	8.7
8		Ala	10.2	Thr	4.3	Phe	7.2	Val	3.1	Ala	6.0
9*				Glu	1.2	Asp	3.2	Glu	3.6	Pro	20.7
10		Leu	18.6	Gly	7.7						
11		Ala	41.4	Gln	4.5						
12				Arg	2.4						
13			Gly 29.4								
14		Glu	11.7								
15			Ala 13.1								
16		Asp	10.2								
17		Glu	6.7	Ala	5.3						
18		Pro	9.5	Ala	1.1						
19		Val	8.9								
20		Ile	8.9	Ala	4.8						
21			Leu 7.3								
22*		Glu	7.9	Ala	6.2						

*Means that the runs were deliberately stopped at the last cycle given.

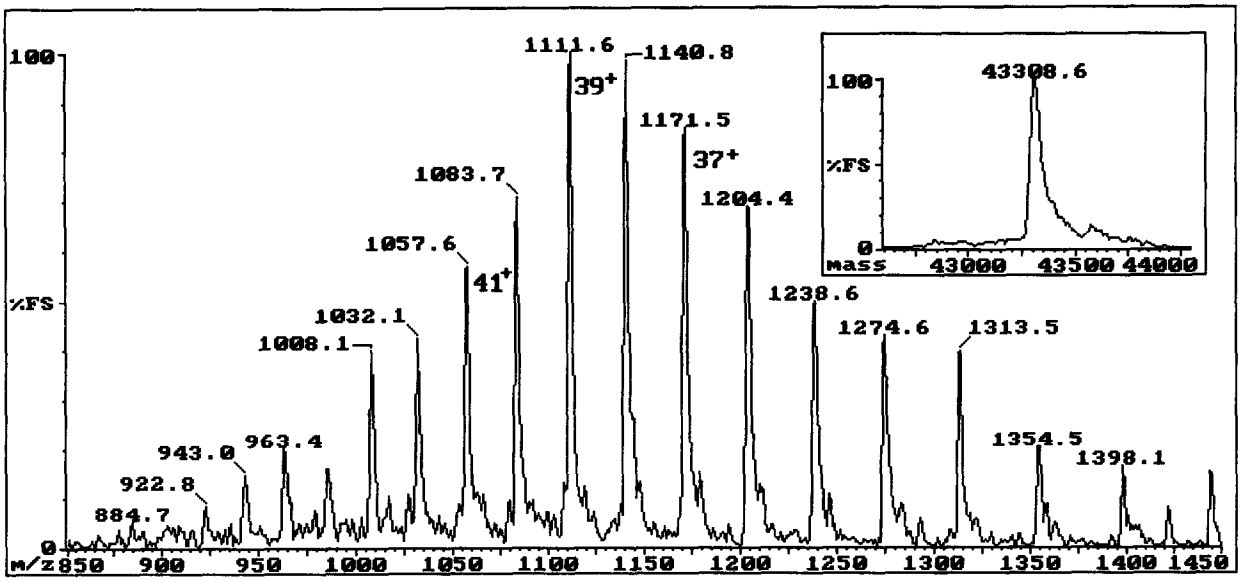

Fig. 2 Electrospray ionisation mass spectrum of a freshly prepared sample of the α subunit of MADH from *T. versutus*. The insert is the molecular mass of the subunit calculated from the different values of *m/z* indicated in the spectrum; the number of charges for each value is followed by the sign '+'.

after the latter had been separated from the β subunit by ultrafiltration through a Centricon filter with a cut-off value of 30 kDa. The resulting spectrum is given in Fig. 2. The different *m/z*-values allow the calculation of a molecular mass of 43,308.6 Da. With an accuracy even better than 0.01%, this result indicates that no other residue than Gln-32 would be the N-terminal residue of the α subunit.

3.3. *Confirmation of the identity of the N-terminal residue*

The indication, on basis of the electrospray mass analysis that Gln-32 would be the N-terminus of the α subunit (and of the MADH in total) would imply an explanation for the absence of any sequence result on band a (Fig. 1(I)). The formation of pyroglutamic acid from glutamine, when the residue is at the N-terminal position, is known to occur frequently in eucaryotic as well as in procaryotic proteins mostly as a result of the isolation procedure of the proteins. In order to test whether this cyclisation had also taken place here, we cut out band a from the second lane of the blotted α subunit and performed an in situ digest with pyroglutamate aminopeptidase. Upon subsequent Edman degradation of digested band a, we now did obtain sequence information. The quantitative data, given in Table I, allowed the deduction of the sequence Thr-Glu-Pro-Ala-Glu-Pro-Glu-Ala-Pro. This is exactly the sequence starting after Gln-32, in accordance with the specificity of the aminopeptidase and proving the identity of residue 32 as being pyroglutamic acid.

From a protein methodology point of view, we like to mention that we are not aware of a succesful application of an in situ digest of an electroblotted and Amido black stained protein band with pyroglutamate aminopeptidase, although such a report may of course have escaped our attention.

To confirm the result, we also carried out a digest in solution of native MADH with Pe-ase and analyzed the effect of the enzyme by N-terminal sequence analysis. As expected, we obtained both the sequence Thr-Glu-Pro-Ala-Glu-Pro-Glu-Ala-Pro from the cleaved α subunit as the sequence Ala-Gly-Pro-Ala-Glu-Gly-Val-Asp-Pro [6] from the β subunit (quantitative data not shown). On these grounds, we unambiguously confirm that the polypeptide of the α subunit of MADH consists of 395 residues and that it covers the region Gln-32–Ser-426 of the coding gene.

The result also means that the real N-terminal region is 25 amino acids longer at the N-terminal side than the sequence seen in the 3-dimensional structure of MADH. This suggests that either the N-terminal region is badly defined and no density found, or the region is not present at all in the crystals due to proteolytic cleavage. We believe the latter to be the case. The N-terminal region is negatively charged with 6 out of 25 amino acids being glutamates which may be highly exposed to the solvent. These may serve as cleavage sites for proteolytic degradation. The chemical instability of the N-terminal region can also be explained by the 3D-structure. The α subunit consists of a compact disc-shaped domain with mainly β-sheets, plus the N-terminal region that forms an extended arm which embraces the β subunit [5]. In the tetramer, this possibly will cause greater stability, but when separated this extended arm will not contribute to the stability of the subunit. The same explanation

can be given for the origin of smaller stable fragments c1 and c2. In the three-dimensional structure, the cleavage site of these fragments is situated in the loop that is very exposed to the solvent [5,9].

Acknowledgements: This work was supported by the Belgian Fund for Joint Basic Research (Contract 32.0018.91) and the Belgian Fund for Medical Scientific Research (Contract 39.0038.91).

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