The Molecular Size of *N*-Methylglutamate Dehydrogenase of *Pseudomonas aminovorans*

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N-Methylglutamate dehydrogenase, purified to a specific activity of 0.29 unit/mg of protein, gave one band on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, corresponding to a molecular weight of 130000. Enzyme–Triton complexes were found to have a partial specific volume of 0.73 cm³/g, suggesting that the protein binds less than 0.1 g of Triton/g of protein. A molecular weight for the intact enzyme in the presence of 1% (w/v) Triton X-100 of 550000 suggested that the enzyme may be a tetramer.

We have previously described the solubilization and partial purification of *N*-methylglutamate dehydrogenase from *Pseudomonas aminovorans* to a state in which ten protein species were detectable by polyacrylamide-gel electrophoresis at pH 8.3 (Bamforth & Large, 1977; see also Bamforth, 1976). We now report an improved purification procedure for the enzyme, yielding material sufficiently close to homogeneity to permit the evaluation of the subunit molecular size of the enzyme. We also present data relevant to the size of the Triton X-100-solubilized enzyme.

Materials and Methods

Materials

Urease, catalase, thyroglobulin, arginine decarboxylase, 1,4-diaminobutane, 1,5-diaminopentane and 1,6-diaminohexane were from Sigma (London) Chemical Co., Kingston-upon-Thames, U.K. Rabbit muscle lactate dehydrogenase was from Boehringer Corp., Lewes, East Sussex, U.K. Sepharose 4B was obtained from Pharmacia (G.B.), London W.5, U.K., and was converted into the 4-aminobutyl, 5-aminopentyl or 6-aminohexyl derivatives as described by Shalteil & Er-El (1973). CNBr was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. Other materials were obtained from the sources previously described (Bamforth & Large, 1977).

Partial enzyme purification

Partially purified *N*-methylglutamate dehydrogenase was prepared as described by Bamforth & Large (1977).

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Chemical determinations

Protein was determined by the method of Wang & Smith (1975).

Enzyme assays

N-Methylglutamate dehydrogenase was assayed as described by Bamforth & Large (1977). Arginine decarboxylase was determined manometrically by the method of Boeker & Snell (1971). Urease and lactate dehydrogenase were determined as described in *Biochemica Information* (1973, 1975), vols. 1 and 2, Boehringer Corp., Mannheim, W. Germany. During the urease assay samples containing Triton X-100 gave a dense precipitate, which necessitated the removal of detergent before assay as previously described (Bamforth & Large, 1977). Catalase was located by measuring the A_{405} against an appropriate buffer blank.

Polyacrylamide-gel electrophoresis

Electrophoresis at pH 8.3 has been described previously (Bamforth & Large, 1977). Electrophoresis at pH 4.3 was performed in the same manner, but by using the solutions described for use at this pH by Reisfeld *et al.* (1962).

SDS†/polyacrylamide gels were prepared as described by Zahler (1974). Standard proteins for subunit molecular-weight determination were from the Boehringer Combithek electrophoresis calibration kit (catalogue no. 15661). Electrophoresis was carried out at 2 mA per tube for 16.5 h at 20°C. The gels were stained with Amido Black and destained as described previously (Bamford & Large, 1977).

† Abbreviation: SDS, sodium dodecyl sulphate.

Results

Further purification of N-methylglutamate dehydrogenase

Further purification of the enzyme was necessary to obtain material of purity suitable for subunit molecular-weight determination.

Partially purified enzyme (56 mg of protein) eluted from a column of hydroxyapatite (Bamforth & Large, 1977) was dialysed against 100 vol. of 50 mM-potassium phosphate, pH 7.0, containing 1% (w/v) Triton X-100 at 4°C for 4 h and was applied at 4°C to a column (2 cm × 6 cm) of DEAE-cellulose (Whatman DE-11) equilibrated in the same buffer. After the column had been washed with this buffer the enzyme was eluted with 100 mM-potassium phosphate, pH 7.0, containing 1% (w/v) Triton X-100. The combined active fractions were pooled and applied at 4°C to a column (1 cm × 3 cm) of 5-aminopentyl-Sepharose 4B, which had been equilibrated with 50 mM-potassium phosphate, pH 7.0, containing 1% (w/v) Triton X-100. The column was then washed with 5 column volumes of this buffer, 5 column volumes of 0.1 M-KCl in the above buffer and then the activity was eluted with 0.2 M-KCl in this buffer. The combined active fractions were stored at -15°C.

The enzyme was found not to bind to 4-aminobutyl-Sepharose 4B under the above conditions, whereas the enzyme did bind to columns of 6-aminohexyl-Sepharose 4B, but only 40% of the activity was subsequently recoverable by elution with KCl.

The effect of the additional two steps is to increase the purification by 2-3-fold over that obtained by using the previously reported procedure (Bamforth & Large, 1977). Enzyme of specific activity 90 munits/mg of protein gave three bands on polyacrylamide-gel electrophoresis at pH 8.3 in the presence of Triton X-100; 76 µg of protein was applied to the gel. The band of lowest mobility was the major component and coincided with the single band of activity detected by using the Nitro Blue Tetrazolium reductase stain (Bamforth & Large, 1977). Similar preparations gave two protein bands on electrophoresis at pH 4.3, both of very low mobility, but activity was undetectable. The enzyme has been shown to be acid-labile (Bamforth & Large, 1977).

The highest specific activity obtained for purified *N*-methylglutamate dehydrogenase was 290 munits/mg of protein; the two inert protein species observed on polyacrylamide gels at pH 8.3 were almost absent from this material.

Yields from the additional purification steps were low (less than 5% of the activity in the crude extract), but the material was suitable for subunit molecular-weight determination on SDS/polyacrylamide gels.

Preparative polyacrylamide-gel electrophoresis was used in an attempt to obtain homogeneous

Fig. 1. Determination of the subunit molecular weight of *N*-methylglutamate dehydrogenase by SDS/polyacrylamide-gel electrophoresis

Electrophoresis was performed as described in the text. ○, Migration positions of the standard proteins: 1, RNA polymerase β' -subunit (165 000); 2, RNA polymerase β -subunit (155 000); 3, bovine serum albumin monomer (68 000); 4, RNA polymerase α -subunit (39 000). ●, Migration position of *N*-methylglutamate dehydrogenase. Values in parentheses denote molecular-weight values assumed. Specific activity of the *N*-methylglutamate dehydrogenase sample used was 290 munits/mg of protein.

enzyme. However, the recovery of active enzyme from homogenized slices of gels was very poor.

Subunit molecular-weight determination

By using the Boehringer Combithek protein markers a subunit molecular weight of $130\,000 \pm 20\,000$ on SDS/polyacrylamide gels was obtained for the only detectable protein band from *N*-methylglutamate dehydrogenase (Fig. 1); 6.3 µg of protein was applied to the gels. An identical value was obtained by using bovine serum albumin monomer and dimer, lactate dehydrogenase, pyruvate kinase and catalase as the protein markers.

Molecular weight of the enzyme

In the presence of 1% (w/v) Triton X-100, the molecular weight of *N*-methylglutamate dehydro-

Fig. 2. Determination of the molecular weight of *N*-methylglutamate dehydrogenase by gel filtration on Sepharose 6B

To a column of Sepharose 6B (2.5cm×75cm), equilibrated with 50mM-potassium phosphate, pH 7.0, containing 1% (w/v) Triton X-100 were applied separately 3mg samples of protein dissolved in the same buffer. During elution at 4°C with this buffer, 4ml fractions were collected and enzyme activities measured as described in the Materials and Methods section. Protein was located by measuring A_{230} (Andrews, 1970), with buffer containing Triton X-100 in the reference cuvette. Arginine decarboxylase and urease were then chromatographed on the same column in 200mM-potassium phosphate, pH 6.0, containing 1% (w/v) Triton X-100, since the former enzyme dissociates at pH values below 6 (Boeker & Snell, 1971). The elution volume (V_e) of urease was not affected by pH. ●, Elution positions of the standard proteins: 1, lactate dehydrogenase (130000); 2, catalase (248000); 3, urease (482000); 4, thyroglobulin (670000); 5, arginine decarboxylase (850000). △, Elution position of *N*-methylglutamate dehydrogenase. Values in parentheses denote molecular-weight values assumed.

genase was determined by its behaviour on gel filtration in Sepharose 6B (Andrews, 1970; Libor *et al.*, 1975) compared with standard calibration proteins (Fig. 2). The molecular weight of *N*-methylglutamate dehydrogenase was about 550000 ± 40000 at pH 7.0.

In a linear gradient of 5–21% (w/v) sucrose in Vol. 167

50mM-potassium phosphate, pH 7.0, containing 1% (w/v) Triton X-100, partially purified *N*-methylglutamate dehydrogenase (Bamforth & Large, 1977) (12.8mg of protein) was centrifuged with 5mg of urease by the method of Martin & Ames (1961). Centrifugation was at 25000 rev./min for 19h at 4°C in a 3×23ml swing-out rotor of an MSE Super-speed 65 preparative ultracentrifuge. After centrifuging, the tube was pierced at the bottom and 0.5ml fractions were collected and assayed. The peak of activity for *N*-methylglutamate dehydrogenase coincided with that for urease.

Discussion

The imposition of ion-exchange and hydrophobic chromatographic steps on the previously reported partial purification procedure (Bamforth & Large, 1977) leads to an almost homogeneous enzyme. In the most purified sample a specific activity of 290 nmol of 2,6-dichlorophenol-indophenol reduced/min per mg of protein was obtained, which represented an overall purification of 145-fold; a visual estimate suggested that the enzyme accounted for a minimum of 90% of the protein seen on polyacrylamide gels at pH 8.3. In conjunction with a subunit molecular weight taken to be 130000, a turnover number of 42 molecules of *N*-methylglutamate oxidized/min per subunit at 25°C is obtained. This compares with a turnover number of 3900 for an apparently comparable enzyme, succinate dehydrogenase (Dervartanian *et al.*, 1966). It should be noted that Tanford & Reynolds (1976) have pointed out the limitations of assuming total dissociation of membrane proteins in SDS solution.

As yet we do not know to what extent Triton X-100 is bound to the enzyme molecule, although it seems certain that the enzyme must bind some detergent, as its removal leads to an immediate aggregation of the enzyme (Bamforth & Large, 1975; Bamforth, 1976). Assuming a molecular weight for the protein-detergent complex of 550000, for the enzyme subunits of 130000 and for Triton X-100 of 647 (Koch-Light Laboratories, Colnbrook, Bucks. SL3 0BZ, U.K.), it can be calculated that monomeric, dimeric, trimeric and tetrameric enzyme would bind approx. 630, 220, 80 and 10 molecules of Triton per subunit respectively to attain the molecular weight of 550000 estimated for the protein-detergent complex.

Clarke (1975) has described a method for estimating molecular weights of detergent-solubilized proteins involving centrifugation of the complexes in $^2\text{H}_2\text{O}$ - and H_2O -based sucrose density gradients. He observes that the method can be used for impure protein preparations in which the amount of detergent bound is unknown, making the assumption that the binding of detergent is identical in

$^2\text{H}_2\text{O}$ and H_2O . Clarke (1975) points out that this assumption does not introduce a large error.

Co-sedimentation of *N*-methylglutamate dehydrogenase with urease suggests a partial specific volume for the protein-detergent complex similar to that of urease, i.e. $0.73\text{cm}^3/\text{g}$ (Sober, 1968), assuming the complex to be spherical. Soluble proteins such as urease bind negligible quantities of detergent (Clarke, 1975) and the presence of detergent does not alter their sedimentation behaviour (Yu *et al.*, 1973). The presence of Triton X-100 ($\bar{v} = 0.94\text{cm}^3/\text{g}$) can be seen not to have substantially altered the partial specific volume of the detergent-free protein (assuming that it has a typical $\bar{v}$ for a protein of $0.71\text{--}0.75\text{cm}^3/\text{g}$). This suggests that the amount of detergent bound by *N*-methylglutamate dehydrogenase is less than 0.1g/g of protein, or less than 21 molecules of detergent per subunit. This is consistent with a tetrameric structure for the enzyme. It should be pointed out that most membrane proteins investigated to date bind between 0.28 and 1.12g of detergent/g of protein (Clarke, 1975). In the absence of a definitive value for the amount of Triton X-100 bound to *N*-methylglutamate dehydrogenase, the conclusion that the enzyme exists as a tetramer in detergent solution must remain speculative.

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