STUDIES ON THE SUBUNIT STRUCTURE OF THE ADENOSYLCOBALAMIN-DEPENDENT ENZYME ETHANOLAMINE AMMONIA-LYASE

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1. Introduction

The clostridial adenosylcobalamin-dependent enzyme ethanolamine ammonia-lyase (EC 4.3.1.7) is reported to have mol. wt 520 000 [1] and to contain 2 active sites/molecule [2] On the basis of results obtained by ultracentrifugation in the presence of guanidinium hydrochloride, it was proposed [1] that the native enzyme molecule is composed of 8–10 subunits of mol. wt 51 000. There was however some evidence of heterogeneity of subunits in these results.

The present investigation was initiated to re-evaluate the subunit structure of the enzyme using sodium dodecyl sulphate (SDS)–acrylamide gel electrophoresis [3]. This technique is a very powerful tool for such studies and is capable of resolving species which cannot be distinguished by the procedure in [1] Two different subunits, mol. wt 51 000 and 36 000, were found in equimolar proportions indicating that one molecule of native enzyme is made up of 12 subunits, 6 of each type.

2. Materials and methods

2 1. *Enzyme purification*

Ethanolamine ammonia-lyase was purified from cultures of *Clostridium* sp. and resolved of bound cobalamins by the method in [4] modified according to [5] Protein was determined by the methods in [6,7] using bovine serum albumin as a standard and the results were corrected by a factor determined from dry weight measurements on purified enzyme protein

2.2. *SDS–acrylamide gel electrophoresis*

SDS–acrylamide gel electrophoresis was carried out as in [3] for SDS–disc and SDS–phos gels on 7 × 0.6 cm cylindrical gels. Gels were stained in Coomassie brilliant blue and were scanned at 540 nm in the densitometer attachment of a Beckman SP 1800 spectrophotometer.

Molecular weight estimations were carried out on SDS–disc gels containing 10% acrylamide with 100 μl 3% spacer gel above. In some experiments 7.5% or 13% acrylamide was used. Duplicate samples were prepared, each in 50 μl 0.05 M Tris–HCl buffer (pH 6.7), 1% SDS, 1% 2-mercaptoethanol, containing 5 μg ethanolamine ammonia-lyase with and without 2.5 μg each of bovine serum albumin, catalase, horse liver alcohol dehydrogenase, carbonic anhydrase and bovine growth hormone. Separate gels were run with the above marker proteins and fumarase and lactate dehydrogenase. All samples were heated for 2 min at 100°C before addition of 5 μg bromophenol blue and a few crystals of sucrose. After running for 4.5–5 h at 2 mA/gel the centre of the bromophenol blue band was marked with a needle dipped in Indian ink and the R_F of each band relative to that of bromophenol blue was measured after staining.

Preparative separation of subunits was accomplished on washed 7.5% SDS–phos gels as in [8]. Ethanolamine ammonia-lyase was precipitated from 0.01 M potassium phosphate buffer (pH 7.4) by addition of 3 vol. acetone. After centrifugation the pellet was dissolved at a final 3.5 mg/ml in 0.01 M sodium phosphate buffer (pH 6.7) containing 3.75% SDS and heated for 2 min at 100°C. Samples, 100 μl, each also containing 5 μg bromophenol blue and a few crystals

of sucrose, were applied to each gel and these were run at 90 V until the bromophenol blue reached the bottom of the gels. Slices cut from the side of each gel were stained for 15 min and destained while the rest of the gels were kept cool. The stained slices were used as guides to cut segments containing the separated subunits from the gels. Protein was eluted by shaking thin slices of the gel segments in about 10 vol. 0.01 M sodium phosphate buffer (pH 6.7), containing 0.1% SDS, for 24 h [8] and the final eluate concentrated in an Amicon Minicon concentrator. Purity of the separated chains was checked by rerunning about 50 μg each polypeptide (determined as in [7]) on 7.5% SDS–phos gels. Preparations containing one chain with no detectable contamination were used further.

2.3. *N-terminal sequence analysis*

Separated chains or native enzyme were precipitated with 3 vol. acetone, ~5 nmol protein being used for determination of each amino acid. N-terminal amino acids were identified by the method for proteins in [9]. The next two amino acids were determined by the SDS–dansyl-Edman procedure [8] except that the new N-terminal amino acid after each step was identified as in [9] after precipitation from coupling buffer with 10 vol. 20% trichloroacetic acid and washing with ether. Dansyl amino acids were separated by thin-layer chromatography on polyamide layers [10] and, after initial identification, each was shown to run as one spot when mixed with an authentic sample of the same dansyl amino acid.

2.4. *Amino acid analysis*

Samples of native enzyme and separated subunits were precipitated in 10% trichloroacetic acid for 30 min. The pellets after centrifugation were washed 3 times with ether, dried and hydrolysed in 6 N HCl for 24 h at 110°C in evacuated sealed tubes. Amino acid analyses were carried out using a Locarte amino acid analyser 1/2 Cystine and tryptophan were not determined.

Amino acid analysis of Coomassie blue-stained bands cut from a washed 7.5% SDS–phos gel after electrophoresis of 60 μg enzyme was as in [11].

2 5. *Chemicals*

Bovine serum albumin, horse liver alcohol dehydrogenase, catalase, fumarase and carbonic anhydrase were obtained from Sigma Chemical Co. Lactate dehydrogenase was from the Boehringer Corporation Ltd. and SDS (sodium lauryl sulphate, specially purified for biochemical work), Coomassie brilliant blue, dansyl chloride, dansyl derivatives of methionine, leucine, isoleucine, phenylalanine and serine and polyamide layer plates were from BDH Chemicals Ltd. Bovine growth hormone was a gift from Dr M. Wallis. Other chemicals were of the best commercial grade available.

3. Results

3.1. *Subunit structure of ethanolamine ammonia-lyase*

Figure 1 shows the presence of 2 well-resolved bands (I and II) when SDS-treated enzyme was subjected to electrophoresis on 7.5% SDS–phos gels. The same results were obtained in the presence and absence of 2-mercaptoethanol indicating the absence of interchain disulphide bridges. No other bands were detected when up to 200 μg protein were run on one gel. Measurement of the area under each peak gives a rough measure of the protein content of each band (but see [3]). The ratio of the area under peak I to that under peak II was found to be 1.43 ± 0.22 (SD)

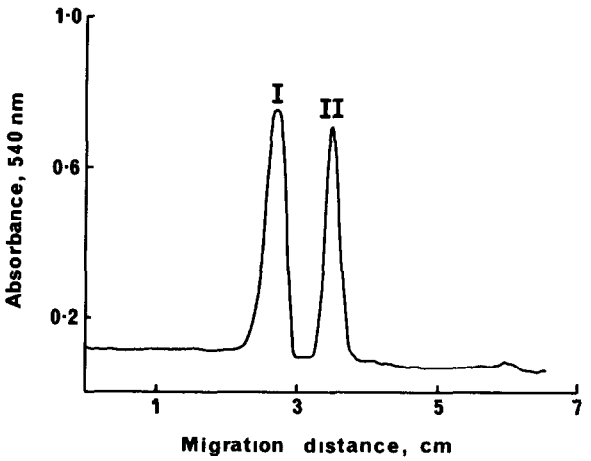

Fig.1 SDS–acrylamide gel electrophoresis of ethanolamine ammonia-lyase 14 μg enzyme was subjected to electrophoresis on a 7.5% SDS–phos gel which was stained and scanned as in section 2

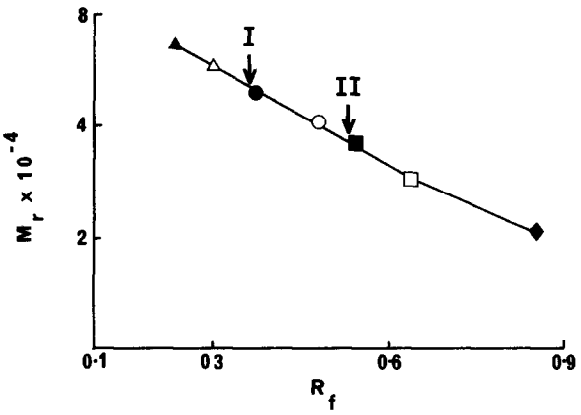

Fig.2 Molecular weight estimation. Ethanolamine ammonia-lyase, bovine serum albumin (▲), catalase (△), fumarase (●), alcohol dehydrogenase (○); lactate dehydrogenase (■), carbonic anhydrase (□), and bovine growth hormone (♦), were subjected to electrophoresis on SDS–disc gels and the gels analysed as described in the text. Results are an average of duplicate runs

from measurements on 16 stained gels utilising enzyme prepared from 4 different batches of clostridia.

3.2. *Molecular weight of subunits*

Figure 2 shows the R_F relative to bromophenol blue on 10% SDS–disc gels of I, II and 7 marker proteins of known molecular weight. Average results from 6 separate experiments indicate app mol. wt 51 000 for I and 36 000 for II by this procedure. Similar molecular weights were obtained in electrophoresis on 7.5% and 13% SDS–disc gels The ratio of the molecular weight of I to that of II is very close (1 42) to that of the protein content of the two bands, as estimated above, indicating that the 2 subunits are probably present in equimolar proportions.

3 3. *N-terminal sequence analysis*

Fluorescent spots corresponding to dansyl-methionine and dansyl-methionine sulphoxide were observed when the whole enzyme or either of the separated chains was subjected to the procedure in [9]. Only dansyl-methionine sulphone was found after performic acid oxidation of the protein. These results indicate that methionine is the N-terminal amino acid for both subunits. After one cycle of SDS–Edman degradation isoleucine was identified as the new N-terminal amino acid of I while phenyl-alanine was that of II (24 h hydrolysis was required to release dansyl-leucine). N-terminal leucine (for I) and serine (for II) were found after a second cycle of degradation. The following N-terminal sequences are therefore indicated·

I Met–Ile–Leu–

II Met–Phe–Ser–

3 4. *Amino acid analysis*

Table 1 shows the results of amino acid analysis carried out on native enzyme and on purified subunits I and II after elution from gels (c.f. [1]). The amino acid composition of the 2 separated chains is similar

Further information on the molar ratio of the two subunits was sought by carrying out amino acid analysis directly on stained bands cut from gels, and

Table 1
Amino acid analysis of native enzyme and separated subunits

Residue	nmol residue/nmol polypeptide[a]			
	Native enzyme	I	II	I + II
Asp	90 6	59.3	37 3	96.6
Thr	49 5	29.0	17.2	46 2
Ser	42 7	20.6	19.9	40.5
Glu	94 3	57.9	38.5	96.4
Pro	31.5	17.1	12.6	29.7
Gly	68.7	41 0	28 8	69 8
Ala	73.9	46 5	27 3	73.8
Val	56 8	29.9	24 1	54.0
Met	23 0	10.9	11.7	22 6
Ile	50.3	29.7	18 6	48 3
Leu	71.8	40.8	32 8	73 6
Tyr	23 5	15.8	8.8	24.6
Phe	23 1	15.7	7.3	23.0
His	15.9	9.0	6.2	15 2
Lys	57.4	30 0	24.6	54.6
Arg	27.6	15.4	13.2	28.6

[a] nmol polypeptide (taken to be 87 μg for native enzyme, i.e , one of each subunit, 51 μg for I and 36 μg for II) were calculated from the total recovery of amino acids, uncorrected for cysteine and tryptophan

Amino acid analysis was carried out as in section 2. Results are the average of 3 determinations for native enzyme and 2 determinations for I and II

thus estimating the relative weights of amino acids in the 2 bands. Some destruction of amino acids was observed; however the ratios of 3 of the most stable amino acids recovered from band I to those from band II were found to be: alanine, 1.82 (1.75); leucine, 1.42 (1.37), isoleucine, 1.36 (1.65). The figures in parentheses give the calculated ratios from equimolar proportions of I and II with the amino acid compositions given in table 1. It can be seen that the results are consistent with the presence of equal numbers of large and small subunits.

4. Discussion

The results described above indicate that ethanolamine ammonia-lyase contains 2 different subunits of app. mol. wt 51 000 and 36 000 in equimolar proportions. Presumably the smaller subunit was not resolved from the 51 000 peak by the procedure in [1] If each molecule of native enzyme is composed of 6 of each type of subunit, it would be mol. wt 522 000 which is in good agreement with the published mol. wt 520 000 [1]. Both subunits were found to have the same N-terminal amino acid, methionine, but to differ in the second and third positions in the chain. The detection of a unique N-terminal sequence for each subunit renders it improbable that more than one type of polypeptide of each molecular weight is present.

It is difficult to reconcile a subunit structure for the native enzyme molecule of $(I_6 + II_6)$ with the presence of 2 active sites/molecule [2] Present work is directed towards a further examination of this problem

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