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COENZYME B-12-DEPENDENT REACTIONS

PART IV *. OBSERVATIONS ON THE PURIFICATION OF ETHANOLAMINE AMMONIA-LYASE

KEITH N. JOBLIN, ALAN W. JOHNSON, MICHAEL F. LAPPERT and O. CARYL WALLIS

School of Molecular Sciences, University of Sussex, Falmer, Brighton, BN1 9QJ (U.K.)

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Summary

Purification of ethanolamine ammonia-lyase (EC 4.3.1.7) from a *Clostridium* sp. grown at the University of Sussex, U.K. and the National Institutes of Health, U.S.A., has been compared and an improved isotopic assay for the enzyme has been developed. Successful purification of this enzyme from Sussex-grown cells requires modification of the published procedure (Kaplan and Stadtman (1968) J. Biol. Chem. 243, 1787—1793) principally a 70% decrease in volume during precipitation with 0.4 M NaCl. This modification also increases the yield from N.I.H.-grown cells. Purified enzyme, resolved of inactive cobalamins, has the same high specific activity from both sources and behaves in the same way on disc gel electrophoresis. Sussex enzyme, before resolution, has less than 20% of the specific activity of unresolved N.I.H. enzyme and contains over 50% more inactive cobalamin. The bound cobalamin from both preparations has been identified as a "base-on" Co^{II} ψ-cobalamin.

Introduction

The continuing interest in the rearrangements catalysed by coenzyme B-12-dependent enzymes requires a dependable supply of the purified enzymes. The purification and properties of ethanolamine ammonia-lyase (EC 4.3.1.7), a clostridial coenzyme B-12-dependent enzyme, have been described by Kaplan and Stadtman [2,3]. We, as well as others (Babior, B.M., personal communication), have encountered difficulty in growing clostridia giving enzyme in good yield and of comparable specific activity. In order to investigate these difficul-

* Ref. 1 is regarded as Part III of this series.

ties, we have grown clostridia at both the National Institutes of Health (N.I.H.), Bethesda, Md., U.S.A. and at the University of Sussex and have compared the purification of ethanolamine ammonia-lyase from the two sources.

Materials

NADH (disodium salt), yeast alcohol dehydrogenase (EC 1.1.1.1) and bovine serum albumin were obtained from Sigma Chemical Co. Streptomycin sulphate was from B.D.H. Ethanolamine for enzyme purification and assay was laboratory reagent grade from B.D.H. and was re-distilled before use. Bacto-tryptone and Bacto-yeast extract were from Difco Laboratories. Coenzyme B-12 was a kind gift from Glaxo Research Laboratories Ltd. and was always passed through a column of Whatman CM cellulose in the dark prior to use. Sephadex G-200 was obtained from Pharmacia. [2-^{14}C]Ethanolamine hydrochloride was obtained from the Radiochemical Centre, Amersham, and was further purified by Dr. M. Wallis using a Locarte amino acid analyser. Instagel was from Packard. All other chemicals were of the highest purity available commercially.

Methods

Growth of the choline-fermenting *Clostridium* sp. isolated by Hayward [4] was carried out as described by Bradbeer [5] except that Trypticase was replaced by Bacto-tryptone and 5.35 g ethanolamine/l of medium were neutralized with sulphuric acid. Spores used for Sussex growths were from the same stock as those used at N.I.H: three separate batches were kindly supplied by Dr. B.M. Babior. For preparation of enzyme, organisms were normally grown in ethanolamine-containing medium from a 5% inoculum of a choline-grown culture. Conditions of growth were kept as constant as possible in the two laboratories, although a 360 l stainless steel fermentor was used at N.I.H. and a 400 l glass fermentor at the University of Sussex. Organisms were normally harvested by centrifugation 90 min after the stationary phase of growth was reached. Dry weight determinations on the packed cell pellets indicated a similar water content in all preparations. After centrifugation the packed cell pellet was frozen and stored in liquid nitrogen until used for enzyme preparation.

Enzyme purification was similar to that described as "modified purification procedure" by Kaplan and Stadtman [2] amended according to B.M. Babior (personal communication). All procedures were carried out at 0—5° C in a dim red light. Sonication (in a 100 w M.S.E. sonifier at maximum intensity), streptomycin sulphate precipitation and ammonium sulphate precipitation were carried out separately on two 100 g batches of frozen cells. The ammonium sulphate pellets from 200 g cells were then combined in either 50 or 15 ml 0.4 M NaCl in Buffer B (0.01 M sodium phosphate 0.01 M ethanolamine hydrochloride, pH 7.4). The suspension was mixed thoroughly, allowed to stand for 10 min and then centrifuged at 75 000 × *g* for 10 min. The pellet was washed successively in 5 and 1 ml 0.4 M NaCl in Buffer B, each time being allowed to stand for 10 min before centrifugation at 75 000 × g for 10 min. The final pellet was resuspended in about 3 ml Buffer A (0.01 M potassium phosphate, 0.01 M ethanolamine hydrochloride, pH 7.4) and dialysed overnight against

one litre volumes of Buffer A (3 changes). The diffusate, after centrifugation for 10 min at 75 000 × g, was applied to a column of Sephadex G-200 (2.2 × 54 cm) which had been equilibrated with Buffer A, and elution with Buffer A was carried out. The major peak, eluting in about 24 ml after buffer volume equivalent to the void volume of the column had passed, was collected and found to be pure by disc gel electrophoresis. A second small peak containing a colourless, low molecular weight protein and comprising about 10% of the protein eluted from the column was completely separated from the main orange coloured peak. We obtained a more complete and reliable separation of the two components using Sephadex G-200 than with Biogel P-200 used by the previous authors. Purified enzyme eluted from the column was resolved of bound cobalamins as described by Kaplan and Stadtman [3] except that the enzyme was dissolved in Buffer A and acidification was with 5% sulphuric acid in 95% saturated ammonium sulphate. The pellet was washed with 5 ml 75% saturated ammonium sulphate in Buffer A and after centrifugation the pellet remaining was suspended in about 2.5 ml Buffer A and dialysed overnight against 1-l volumes of Buffer A (3 changes). Any denatured protein was removed by centtrifugation for 10 min at 75 000 × *g*. Enzyme was stored in liquid nitrogen before use.

Enzyme assay. Enzyme activity in the crude extract and in the streptomycin sulphate supernatant was determined by an isotopic assay based on that of Bradbeer [5]. As the activity in some preparations was very low it was essential to reduce the background to a low level to obtain meaningful results. This was achieved by the following procedure using very pure [^{14}C]ethanolamine. The assay system contained 30 μmol potassium phosphate, pH 7.4, 21—23 μmol and 0.04 μCi [2-^{14}C]ethanolamine hydrochloride, 80 μmol sodium bisulphite (adjusted to pH 7.4 with KOH), 0.01 μmol coenzyme B-12 and enzyme in a final volume of 0.6 ml. The reaction was started by addition of enzyme sample (0.05—0.75 units) previously kept in ice. Incubation was at 24° C in darkness and samples were taken, in duplicate, at 5 min intervals for 20 min. The reaction was stopped by addition of 100 μl 7.25 M acetic acid and a 0.6 ml sample was removed and added to 0.4 g dry Dowex 50W X8. After 15 min shaking, 0.65 ml distilled water was added and Dowex and precipitated protein removed by centrifugation for 5 min in a bench centrifuge. A 0.7 ml sample of the supernatant was added to 0.2 g dry Dowex 50 and after 30 min shaking 0.35 ml was removed for counting in Instagel in a Packard Tri-Carb Scintillation Counter. Less than 0.03% of the radioactivity added remained in the supernatant after the above Dowex treatment. Acetaldehyde production remained linear for at least 15—20 min in this system. All assays were carried out as soon as possible after preparation of the enzyme sample, normally within 30 min.

Enzyme activity in fractions obtained after ammonium sulphate precipitation was determined by the spectrophotometric assay described by Kaplan and Stadtman [2] again at 24° C. It was found that the activity measured in samples of purified enzyme by either the isotopic or the spectrophotometric assay was the same, one unit of ethanolamine deaminase activity being defined as the amount required to catalyse the conversion of 1 μmol of ethanolamine to acetaldehyde per min.

Protein determination was by the biuret method [6] in fractions from the

early stages in purification and by the method of Lowry et al. [7] in fractions obtained after sodium chloride precipitation. Crystalline bovine serum albumin was used as standard for both assay methods and the results of Lowry determinations on enzyme fractions were corrected by multiplying by a factor of 1.54 [2].

Optical spectra were obtained using a Cary 14 spectrophotometer.

EPR techniques were as described by Lowe et al. [8].

Disc gel electrophoresis was carried out in separating gels (7.5%) at a running pH of 8.5 [9].

Results

Table I shows a comparison of typical results obtained during enzyme purification from organisms grown at N.I.H. (N.I.H. cells) and those grown at the University of Sussex (Sussex cells). The results with N.I.H. cells are in good agreement with those reported by Kaplan and Stadtman [2]. At each step in purification prior to resolution to apoenzyme the specific activity of enzyme from Sussex cells was much lower than that from N.I.H. cells. Removal of bound cobalamins from the N.I.H. enzyme resulted in a 2.4-fold increase in specific activity. In seven other preparations from N.I.H. cells the increase in activity on resolution varied from 1.9 to 3.4-fold (average 2.7-fold) and the final specific activity of resolved enzyme varied from 24 to 34 units/mg protein. The higher specific activity was consistently found in later preparations, presumably due to improved experimental technique. A much greater increase

TABLE I

PURIFICATION OF ETHANOLAMINE AMMONIA-LYASE FROM N.I.H. AND SUSSEX CELLS

Enzyme purification from 200 g (wet weight) cells was carried out as described in the text. 50 ml 0.4 M NaCl in Buffer B was used in the first extraction with N.I.H. cells and 15 ml with Sussex cells. Results are corrected for losses in sampling.

Fraction	Total enzyme activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
N.I.H. Cells				
Crude extract	4 980	14 700	0.34	100
Streptomycin supernatant	4 140	10 900	0.38	83
0.4 M NaCl pellet	1 090	92	12	22
Sephadex G-200	848	60	14	17
Resolved enzyme	1 730	51	34	—
Sussex Cells				
Crude extract	524	6 320	0.083	100
Streptomycin supernatant	513	6 340	0.081	98
0.4 M NaCl pellet	66	32	2.1	13
Sephadex G-200	35	23	1.5	6.7
Resolved enzyme	560	16	35	—

in specific activity on resolution to apoenzyme was found with Sussex enzyme (23-fold in Table I) so that the specific activity of resolved enzyme from both preparations was not significantly different. The average increase for four Sussex preparations was 17-fold and the final specific activity varied from 23 to 35 units/mg protein.

Disc gel electrophoresis was carried out on the sodium chloride-insoluble fraction, the major peak eluted from the Sephadex G-200 column, and the purified resolved enzyme. In both preparations the results were similar to those of Kaplan and Stadtman [2], a major band remaining near the top of the gel in all fractions and a minor faster running band being found only before column chromatography.

The yield of enzyme from 200 g (wet weight) of cells has been consistently low for Sussex cells (approximately 30% of that from the same weight of N.I.H. cells). In the Sussex purification noted in Table I, the protein content of the crude extract was also low but this was not always the case, release of protein during sonication being more variable for Sussex cells than for N.I.H. cells. The modified purification procedure of Kaplan and Stadtman [2] which we followed is based on the observation that ethanolamine ammonia-lyase has an unusually low solubility in solutions of high ionic strength and is thus almost selectively precipitated in 0.4 M NaCl. In our initial attempts to follow their procedure with Sussex bacteria, no protein was precipitated in 0.4 M NaCl presumably because of the low enzyme concentration in the preparations. It was necessary to reduce the volume of the first NaCl wash to 15 ml with Sussex cells to precipitate any enzyme. Use of this lower volume of 0.4 M NaCl also improved the yield of enzyme from N.I.H. cells without altering its purity.

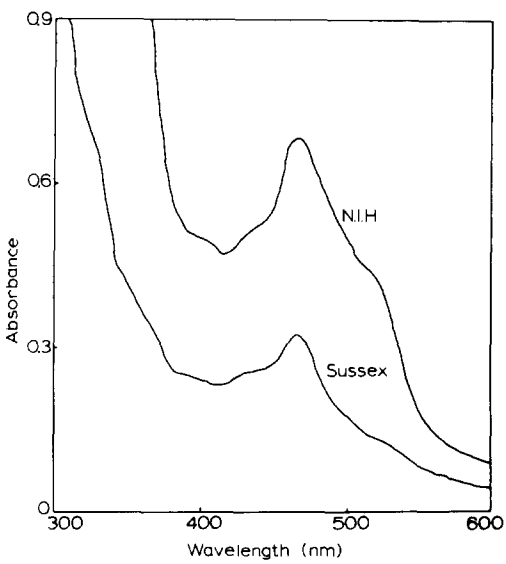

Fig. 1. Optical spectra of ethanolamine ammonia-lyase before resolution of bound cobalamins. Samples were the sodium chloride insoluble fractions of Sussex and N.I.H. cells (the preparations shown in Table I) after dialysis against Buffer A and centrifugation as described in the text. The protein concentrations were 7.5 mg/ml (Sussex) and 24 mg/ml (N.I.H.).

Further modifications such as longer sonication times, disruption of the organisms using a French Pressure Cell or more selective ammonium sulphate fractionation prior to the 0.4 M NaCl precipitation, failed to increase the yield of enzyme from Sussex cells.

EPR spectroscopy and optical spectroscopy indicate that the nature of the cobalamin bound to the native Sussex enzyme is the same as that bound to the native N.I.H. enzyme. The optical spectrum (Fig. 1) is that of a Co^{II} species without any contribution from the hydroxocobalamin witnessed by Kaplan and Stadtman in their preparations. The EPR investigation (Fig. 2) shows that this Co^{II} species consists of two Co^{II} cobalamins, one a normal B-12r-like species and the other a superoxocobalamin. Furthermore, the presence of superhyperfine coupling to the Co^{II} signal in the g_z portion of the EPR spectrum confirms that the Co^{II} species has a nitrogen base coordinated in the axial position, i.e. is "base-on". In order to identify the axial base of the Co^{II} species bound to the Sussex enzyme, the procedure used by Kaplan and Stadtman [3] to identify the cobalamin bound to the N.I.H. enzyme was applied. This indicated that in both the Sussex and the N.I.H. enzymes the cobalamin was a Co^{II} ψ-cobalamin.

The concentration of cobalamin bound to the enzyme was calculated from the optical spectra, assuming that the molecular extinction coefficient at 473 nm of the bound cobalamin was that of B-12r in aqueous solution ($0.92 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) [10]. Results were corrected for absorption by the protein at this wavelength and the enzyme concentration (corrected for the presence of the low molecular weight contaminant) was calculated using a molecular weight of 520 000 [3]. There are two active sites per molecule [11]. Results indicated that in the preparation shown in Table I, 1.3 mol cobalamin were bound per mol N.I.H. enzyme while 2.2 moles were bound per mol Sussex enzyme. In

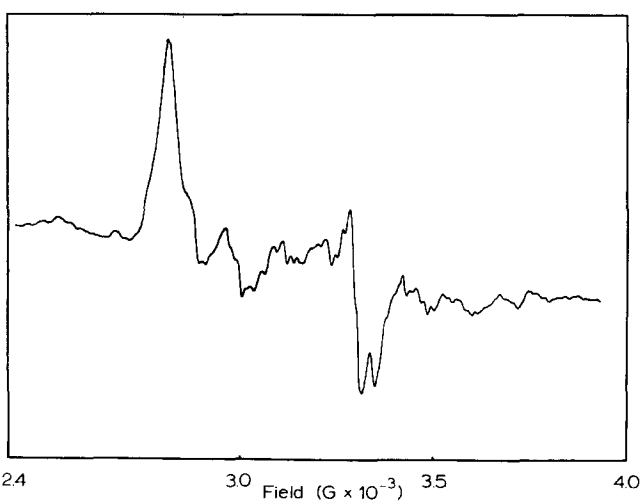

Fig. 2. EPR spectrum of ethanolamine ammonia-lyase before resolution of bound cobalamins. The enzyme was the sodium chloride-insoluble fraction from Sussex cells after dialysis against Buffer A and centrifugation as described in the text. The protein concentration was 21 mg/ml. The spectrum was recorded at 100 K and 150 mW microwave power using 6.3 g modulating field at 100K Hz. The field scale corresponds to a microwave frequency of 9.30.

another pair of preparations 1.6 mol cobalamin were bound per mol N.I.H. enzyme and 2.5 mol per mol Sussex enzyme. Thus, over 50% more cobalamin is bound to Sussex enzyme than to N.I.H. enzyme. The Co^{II} cobalamin is tightly bound and cannot be removed by the gel filtration method of Toraya et al. [12].

The effect of some changes in growth conditions on specific activity of enzyme from Sussex cells was investigated. It should be noted that the growth rate of the organism was similar at N.I.H. and at Sussex and the final yield of cells was comparable. Use of glass-distilled water instead of the usual deionised water or supplementation of growth medium with trace metals (Mn, Co, Mo, Cu) or with sodium selenite did not alter the low specific activity of unresolved enzyme from Sussex cells. When trace metals (Mn, Co, Mo) were added during growth of the organism at N.I.H., enzyme with the normal high specific activity was obtained. Alteration in growth temperature from 30 to 37° C had no effect on enzyme activity, neither did addition of 5% CO_2 to the nitrogen bubbled through the culture, although more reliable growth was obtained in the presence of CO_2. Coenzyme B-12 is light-sensitive and cultures at N.I.H. were grown in the dark in a stainless steel fermentor while those at Sussex were grown in the light in a glass fermentor. However, no difference in enzyme activity was found in preparations from cells grown and harvested in light or darkness at Sussex.

Discussion

Our results indicate that with a minor but important modification in the published purification procedure [2] it is possible to obtain resolved ethanolamine ammonia-lyase of high specific activity from *Clostridium* sp. grown at the University of Sussex. Previous difficulties can be explained by a lower enzyme content in Sussex cells and a markedly reduced specific activity of native enzyme before resolution of bound cobalamins. If the volume of 0.4 M NaCl is decreased, a reasonable yield of purified enzyme is obtained, the full activity of which is realised only in the final resolution step. We have not determined the reason for the difference between cells grown in the two laboratories. Growth in the light or a simple deficiency of trace metals or selenium does not appear to explain the results. Similar results were obtained with clostridia grown from three separate batches of spores sent to us from N.I.H. indicating that a change in the organism is unlikely. Possibly there is a difference in a trace contaminant in one of the components of the medium which further work might identify.

The lower specific activity of native Sussex enzyme is associated with a higher cobalamin content than that of native N.I.H. enzyme. On average 1.2 molecules of cobalamin were bound per active site of Sussex enzyme and 0.7 molecules per active site of N.I.H. enzyme: in the same preparations the average specific activity of native enzyme was respectively 6 and 37% that of resolved enzyme. It seems probable that the low activity of the native enzymes is caused by occupation of the active sites by inactive Co^{II} cobalamin, although binding at an allosteric site cannot be ruled out. A little non-specific binding probably occurs. Once resolved, the enzyme prepared from Sussex cells has the same spe-

cific activity as that prepared from N.I.H. cells and behaves in the same way on disc gel electrophoresis, indicating that the difference in the two preparations lies in the quantity of bound inactive cobalamin and not in the nature of the protein.

EPR and optical spectroscopy show that there is no qualitative difference between the cobalamins bound to the two enzymes. In both cases the orange coloured component has been identified as a Co^{II} ψ-cobalamin which generally contains a small amount of its adduct with molecular oxygen, the derived superoxocobalamin. The presence of a stable enzyme bound B-12r in the native enzyme shows that the apoenzyme can protect the B-12r from reacting with oxygen (a very facile reaction) even during aerobic handling. Presumably some unfolding of the apoenzyme during purification enables the Co^{II} to react with oxygen to form the superoxocobalamin. The origin of the inactive Co^{II} cobalamin bound to the native enzyme in these enzyme preparations is unknown. It may result from the degradation of bound coenzyme during purification or as a consequence of catalysis of substrate [13,14]. Alternatively, it may be present in vivo. It has been shown (Joblin, K.N., Dalton, H. and Lowe, D.J., unpublished results) that native methylmalonyl-CoA mutase from *Veillonella alcalescens* also contains a stable enzyme-bound Co^{II} cobalamin.

The usual requirement in a study of the enzyme reactions mediated by coenzyme B-12 is a supply of the apoenzyme. Positive identification of the bound cobalamin as B-12r means that the purification procedure [2] also provides a convenient source of native Co^{II} holoenzyme and this may prove to be of use in such studies. There is very good evidence [13,14] for enzyme bound Co^{II} being a catalytic intermediate in the rearrangement of substrates for coenzyme B-12-dependent enzymes and, therefore, it would be of interest to transform enzyme-bound Co^{II} to enzyme-bound coenzyme. However, our preliminary attempts to react either 5′-chloro-5′-deoxyadenosine or 5′-deoxyadenosine (a postulated reaction intermediate [15] with this holoenzyme in the presence or absence of substrate have proved negative.

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