Microbial Metabolism of Amino Alcohols

FORMATION OF COENZYME B₁₂-DEPENDENT ETHANOLAMINE AMMONIA-LYASE AND ITS CONCERTED INDUCTION IN ESCHERICHIA COLI

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1. Kinetic studies of ethanolamine ammonia-lyase formation by Escherichia coli suggested that coenzyme B₁₂ (5'-deoxyadenosylcobalamin), with ethanolamine, is a co-inducer. 2. Enzymic and immunological tests failed to show the formation of complementary enzyme components induced separately by ethanolamine and cobalamin respectively. 3. Although specific for ethanolamine as the substrate, enzyme formation was induced by certain analogues, e.g. 2-aminopropan-1-ol. 4. Experiments with cyanocobalamin suggested that neither coenzyme B₁₂ nor some more tightly bound coenzymically inactive cobamide was necessary for enzyme stability in vitro. 5. Mutants of E. coli were obtained which formed ethanolamine ammonia-lyase apoenzyme constitutively, showing that neither ethanolamine nor cobalamin was required for assembly or post-transcriptional stability of the enzyme in vivo. Constitutive enzyme formation was subject to catabolite repression, particularly by glucose. 6. It appears likely that ethanolamine and coenzyme B₁₂, acting in concert, induce ethanolamine ammonia-lyase formation. The term 'concerted' induction is proposed for this phenomenon.

Initial studies of the ethanolamine ammonia-lyase of Escherichia coli showed that its formation required the presence of both ethanolamine and a cobalamin derivative during growth. The phenomenon was termed concerted or 'multivalent' induction (Blackwell et al., 1977). Although both compounds could be required as co-inducers, the possibility was considered that enzyme formation was induced by its substrate, but that the cobalamin was required to ensure the post-transcriptional stability of the enzyme (Blackwell et al., 1977). The purification of ethanolamine ammonia-lyase apoenzyme to apparent homogeneity (Blackwell & Turner, 1978) suggested that coenzyme B₁₂ (5'-deoxyadenosylcobalamin) was not necessary for enzyme stability, although the presence of coenzymically inactive cobalamins was not excluded.

We describe here how ethanolamine ammonia-lyase apoenzyme is stable in the absence of both its substrate and cobalamin compounds in vitro and in vivo and describe experiments supporting the concept of concerted induction of enzyme formation.

Materials and Methods

Micro-organisms, media and enzyme assays

Escherichia coli type I wild-type (N.C.I.B. 8114), its derived mutant strains and the other bacteria

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(1.0 μCi; 8 μg/litre). Bacteria were harvested when the $A_{0.45cm}^{105}$ of the culture was just less than 1.0. Extracts were prepared, as indicated above, and the apoenzyme was purified by a modification of the method of Blackwell et al. (1977) as described by Blackwell & Turner (1978). The procedure involved charcoal treatment of cell-free extracts, fractionation with (NH$_4$)$_2$SO$_4$, DEAE-cellulose chromatography and gel filtration on Bio-Gel A-5m agarose.

The γ-radioactivity corresponding to $^{57}$Co in enzyme fractions was measured at each stage of the purification in a Panax LC-35 lead-castle Geiger counter linked to a Panax P7502A scaler (Panax Equipment, Redhill, Surrey, U.K.). Samples were counted for radioactivity in triplicate and the efficiency (about 82%) was calculated by using a 0.02 μCi $^{137}$Cs standard.

Resolution of tightly bound $[^{57}Co]$cobamide from purified enzyme

Samples of γ-radioactive ethanolamine ammonia-lyase, purified from E. coli grown with cyanob$^{[57}Co]$cobalamin, were precipitated with (NH$_4$)$_2$SO$_4$, under acid conditions by a procedure shown by Kaplan & Stadtman (1968b) to resolve the clastroidial enzyme. Pure enzyme (0.10 mg/ml) in 0.1M-Tris/HCl, pH 7.5, containing 20% (v/v) glycerol, 10 mM-ethanolamine and 4 mM-dithiothreitol (supplemented buffer), was precipitated with 47.6 g of (NH$_4$)$_2$SO$_4$ added to 100 ml of solution at 0°C. Dilute HCl was added dropwise, acidifying the mixture to pH 3. The precipitate was centrifuged at 15000 g for 10 min and washed with 75% (w/v) (NH$_4$)$_2$SO$_4$ in supplemented buffer. The precipitate was then dissolved in supplemented buffer to give a concentration of approx. 0.4 mg of protein/ml. Additional details are given in the legend for Table 2.

Isolation of mutants forming ethanolamine ammonia-lyase constitutively

A culture of wild-type E. coli (N.C.I.B. 8114) was grown on glycerol plus mineral-salts medium, supplemented with ethanolamine plus vitamin B$_{12}$. When the $A_{0.45cm}^{105}$ of the culture was 1.15, the bacteria in 10 ml of culture were harvested aseptically by centrifuging at 5000 g for 20 min and resuspended in 5 ml of sterile 0.1 M-potassium phosphate buffer, pH 7.0. To this suspension was added 0.25 ml of ethyl methanesulphonate (carcinogen), the mixture being incubated with swirling at 37°C for 10 min (Loveless & Howarth, 1959). A 1 ml sample was then used to inoculate 2.5 litres of glycerol plus mineral salts plus ethanolamine plus vitamin B$_{12}$ medium in which growth was limited by the concentration of ethanolamine (35 mg of N/litre), contained in a BioTec FL 103-05 Laboratory Fermentor (BioTec AB, Stockholm 16, Sweden). The fermentor, equipped with standard accessories for agitation, aeration, temperature control and the addition of medium, was operated at 30°C in the batch mode until the culture was fully grown ($A_{0.45cm}^{105}=0.8$). Fresh medium was then added at a constant dilution rate of 0.06/h. During continuous culture ($A_{0.45cm}^{105}=0.80$ at equilibrium), samples were withdrawn, serially diluted and plated on agar-solidified inducing medium. After growth for 48 h, individual colonies were selected for liquid culture on non-inducing medium containing (NH$_4$)$_2$SO$_4$ and later on the same medium supplemented with ethanolamine, vitamin B$_{12}$ or both. Cultures were harvested, extracts prepared and both enzyme activity and immunological cross-reactions tested by the standard procedures.

Immunological methods

Rabbit antiserum to ethanolamine ammonia-lyase from E. coli was obtained as previously described (Blackwell & Turner, 1978). Reactions between serum antibody and enzyme preparations were detected by the Ouchterlony (1949) double-diffusion method, as also described previously (Blackwell & Turner, 1978).

Chemicals

The sources of most chemicals were those reported previously (Blackwell & Turner, 1978). Cyanob$^{[57}Co]$cobalamin was from The Radiochemical Centre, Amersham, Bucks., U.K. Ethyl methanesulphonate was from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. All other chemicals were the purest available from BDH Chemicals, Poole, Dorset, U.K.

Results

Kinetics of induced ethanolamine ammonia-lyase formation

Measurement of the differential rate of [1$^4$C]-ethanolamine incorporation into E. coli (N.C.I.B. 8114) during growth (see the Materials and Methods section) indicated that enzyme synthesis was initiated shortly after the addition of ethanolamine to cultures growing on non-inducing medium supplemented with vitamin B$_{12}$ (cyanocobalamin). A longer lag before incorporation was observed when cyanocobalamin was added to cultures already containing ethanolamine, but not when coenzyme B$_{12}$ (5'-deoxyadenosylcobalamin) was added (Fig. 1a). Similar results were obtained with Klebsiella aerogenes (N.C.I.B. 8267). In E. coli K12 (N.C.I.B. 9484) cyanocobalamin induced enzyme formation as rapidly as 5'-deoxyadenosylcobalamin (Fig. 1b).
These results suggested that the coenzyme form of vitamin B₁₂ was concerned with enzyme induction and that coenzyme formation by *E. coli* K₁₂ was relatively rapid.

**Specificity of substrate-induced enzyme formation**

1-2-Aminopropan-1-ol, added as a supplement (1g/litre) to glycerol plus (NH₄)₂SO₄ plus vitamin B₁₂ medium, induced ammonia-lyase formation to give extracts with about 30% of the activity found when ethanolamine was the inducer. A re-examination of the effect of DL-1-aminopropan-2-ol (Blackwell et al., 1977) showed that it too served as an inducer, albeit a poor one (17% as effective as ethanolamine). The immunological test for enzyme formation, i.e. formation of precipitin lines between antiserum to the enzyme and cell-free extracts during double diffusion in agar (see the Materials and Methods section), confirmed that both higher homologues of ethanolamine acted as inducers. None of the other analogues and derivatives of ethanolamine previously tested as enzyme inhibitors (Blackwell et al., 1977) acted as inducers. In all cases vitamin B₁₂ was required as a co-inducer of enzyme formation during growth.

Neither of the above aminopropanols nor 1,3-di-aminopropan-2-ol diminished ethanolamine-induced enzyme formation when both ethanolamine and the analogue were present at 5mM during growth.

**Search for separately induced enzyme components**

The possibility was tested that the substrate and coenzyme of ethanolamine ammonia-lyase were both required for apoenzyme formation because each induced the formation of a different complementary component of the high-molecular-weight complex (Blackwell & Turner, 1978). When extracts of *E. coli* grown on non-inducing medium supplemented with ethanolamine in one case and vitamin B₁₂ in the other were mixed and incubated under a variety of conditions, no development of enzyme activity was found.

Immunological tests also failed to detect the formation of any material cross-reacting with antiserum to the enzyme in extracts of *E. coli* grown on non-inducing medium supplemented with either ethanolamine or vitamin B₁₂. Strong cross-reaction was seen in control experiments with extracts of *E. coli* grown on medium supplemented with both co-inducers.
Tightly bound cobamide in purified enzyme preparations

Although apoenzyme preparations, purified to apparent homogeneity, had been shown to possess a residual activity in the absence of exogenously added cofactor of less than 2% of that found in its presence (Blackwell & Turner, 1978), the possibility remained that a cobamide was present in significant amounts, but unable to act as a coenzyme. This was supported by the observation that concentrated solutions of the enzyme (over 15 mg of protein/ml) were characteristically orange in colour at pH 7.5. The absorption spectra of enzyme preparations also suggested the presence of cobamide (C. M. Blackwell & J. M. Turner, unpublished work).

To test the possibility that a tightly bound cobamide was playing a structure-stabilizing rather than a coenzymic role, E. coli was grown on media containing cyano$^{57}$Co-cobalamin (see the Materials and Methods section) and the ethanolamine ammonia-lyase was purified from extracts by the procedure described previously (Blackwell & Turner, 1978). It was found that, although most bound cobalamin derivatives were removed at an early stage by charcoal treatment, $\gamma$-radioactivity was associated with the apoenzyme at each stage of purification (Table 1). The association of radioactivity with enzyme activity during the final stage of purification by gel filtration is shown in Fig. 2. From the specific radioactivity of the cyano$^{57}$Co-cobalamin used and assuming a minimum molecular weight for the apoenzyme of 560,400 (Blackwell & Turner, 1978), it was calculated that about 3.2 mol of cob-
amid was bound per mol of apoenzyme. Gel filtration by the procedure of Toraya et al. (1971) failed to remove enzyme-bound cobamide, but precipitation with (NH₄)₂SO₄ and acid treatment (see the Materials and Methods section) resulted in the loss of most of the ⁵⁷Co radioactivity. In contrast, less than one-fifth of the original enzyme activity was lost (Table 2), indicating that the more tightly bound cobamide was not essential for structural integrity and stability. The amount of γ-γ-radioactivity remaining in the preparation corresponded to only 0.64 mol of cobamide/mol of apoenzyme. The low-cobamide-content apoenzyme could be stored, in buffer supplemented with ethanolamine, dithiothreitol, glycerol and KCl (Blackwell & Turner, 1978), for several weeks at -20°C without appreciable loss of activity measured in the presence of coenzyme B₁₂. These results suggested that neither loosely bound coenzyme B₁₂ nor the tightly bound cobamide was necessary for enzyme stability in vitro.

Constitutive formation of ethanolamine ammonia-lyase

Mutants of E. coli were isolated in which ethanolamine ammonia-lyase formation was constitutive. This was designed to test the possibility that the apoenzyme was stable in vivo, in the absence of coenzymatic or other forms of vitamin B₁₂. The role of vitamin B₁₂ as a co-inducer of apoenzyme formation would thus be clarified. The use of continuous culture, in which the concentration of ethanolamine was growth-limiting (see the Materials and Methods section), yielded mutants possessing high constitutively formed enzyme activity on each occasion when the method was used. At a dilution rate of 0.06/h, cultures became populated predominantly with the mutant strain after about 2 weeks. Two mutant strains of E. coli N.C.I.B. 8114, now deposited with the National Collection of Industrial Bacteria as N.C.I.B. 11361 and N.C.I.B. 11362, were isolated from cultures treated with ethyl methanesulphonate.

Each constitutive mutant possessed high ethanolamine ammonia-lyase activity (400-500 nmol/min per mg of protein) in extracts of the bacteria grown on non-inducing medium after several transfers on media completely devoid of both ethanolamine and cobamide compounds. The specific activity of the enzyme in the induced wild type has been reported to be 100–230 nmol/min per mg of protein (Scarlett & Turner, 1976). Enzyme activity cannot be detected in extracts of the non-induced wild type. Extracts of the mutant strains strongly cross-reacted with antiserum to the purified enzyme. In both cases twin precipitin lines were observed similar to those observed with extracts of the wild type grown on inducing medium (Blackwell & Turner, 1978). Control experiments showed that extracts of the wild type grown on non-inducing medium gave no reaction with the antiserum.

The effect of growth conditions on the ethanolamine ammonia-lyase activity of the constitutive mutant N.C.I.B. 11361 was investigated in detail. This strain was grown on a variety of substrates, each serving as the sole source of carbon and energy, with (NH₄)₂SO₄ as the sole or major nitrogen source. As with the wild type (Blackwell et al., 1977), enzyme formation was regulated by catabolite repression, particularly by glucose and other sugars (Table 3).

A large number of mutants was isolated which were resistant to DL-2-aminopropan-1-ol, a potent inhibitor of ethanolamine ammonia-lyase in E. coli (Blackwell & Turner, 1978). Enzyme activity in each of over 60 such mutants tested was within the normal range for the wild type and it appeared that mutation had affected the selectivity of amino alcohol uptake rather than enzyme production.

Table 2. Effect of treatment with (NH₄)₂SO₄ and acid on the radioactivity of ethanolamine ammonia-lyase apoenzyme corresponding to ⁵⁷Co-cobalamin

<table>
<thead>
<tr>
<th>Sample</th>
<th>⁵⁷Co radioactivity (c.p.m.)</th>
<th>Enzyme activity (units/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated enzyme</td>
<td>2459</td>
<td>5.16</td>
</tr>
<tr>
<td>Precipitated enzyme</td>
<td>395</td>
<td>4.17</td>
</tr>
<tr>
<td>Supernatant after pptn.</td>
<td>1589</td>
<td>0</td>
</tr>
</tbody>
</table>

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Discussion

The main conclusion to be drawn from the results described is that two inducers are required, acting in concert, for the formation of ethanolamine ammonia-lyase in E. coli. The possibility that vitamin B₁₂ was required because coenzyme B₁₂ was necessary for the assembly or stabilization of the oligomeric enzyme (Blackwell & Turner, 1978) was ruled out. Spectrophotometric assays of enzyme activity initiated by the addition of excess coenzyme B₁₂ gave no indication of a time-dependent conformational change. Enzymically inactive apoenzyme
Table 3. Effect of growth conditions on ethanolamine ammonia-lyase formation by E. coli mutant forming the enzyme constitutively

<table>
<thead>
<tr>
<th>Carbon source for growth (1 g/litre of medium)</th>
<th>Ethanolamine ammonia-lyase activity in cell-free extracts (nmol of acetaldehyde formed/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate</td>
<td>Expt. 1: 1010  Expt. 2: 1050</td>
</tr>
<tr>
<td>Fumarate</td>
<td>1000</td>
</tr>
<tr>
<td>Succinate</td>
<td>810</td>
</tr>
<tr>
<td>Alanine</td>
<td>720</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>858</td>
</tr>
<tr>
<td>Maltose</td>
<td>522</td>
</tr>
<tr>
<td>Glycerol</td>
<td>414</td>
</tr>
<tr>
<td>Lactate</td>
<td>285</td>
</tr>
<tr>
<td>Glucose</td>
<td>220</td>
</tr>
<tr>
<td>Sucrose</td>
<td>75</td>
</tr>
</tbody>
</table>

was stable during storage, confirming results obtained with partially purified preparations (Blackwell et al., 1977), and a tightly bound cobamide could be largely removed without significant loss of enzyme activity. After treatment with \[^{35}\text{Co}\]cobalamin-labelled enzyme, approx. 0.64 mol of cobamide/mol of apoenzyme was found. The oligomeric clostridial enzyme, of molecular weight similar to that of *E. coli* (Kaplan & Stadtman, 1968b; Blackwell & Turner, 1978), is known to possess two active sites per molecule of enzyme (Mauck et al., 1975). It therefore seemed unlikely that the small amount of tightly bound cobamide present in the treated enzyme played any structural role during assembly, or stabilized the structure, of the enzyme in *E. coli*. This was confirmed by the isolation of mutants forming ethanolamine ammonia-lyase apoenzyme constitutively. Extracts of these bacteria, grown in the complete absence of both vitamin B\(_{12}\) and ethanolamine, were without enzyme activity unless coenzyme B\(_{12}\) was added to the extracts when high activity was exhibited. This clearly demonstrated that cobamide-free apoenzyme was synthesized by the bacteria, that it was stable in vitro and was readily activated in vivo by the addition of coenzyme.

The studies of inducer specificity showed that certain homologues of ethanolamine were capable of acting as inducers but less effectively. This contrasted with the apparently absolute substrate specificity (Blackwell & Turner, 1978), although it was notable that homologues active as inducers were potent inhibitors of enzyme activity. The kinetic studies of enzyme formation suggested that coenzyme B\(_{12}\) was the active co-inducer. Whereas a lag in enzyme formation occurred when the vitamin rather than the coenzyme was used during growth of *E. coli* type I, there was no lag with the *E. coli* K12 strain. It seems likely that the coenzyme B\(_{12}\) synthetase system (Brady et al., 1962; Peterkofsky & Weissbach, 1963, 1964; Vitols et al., 1966) is formed constitutively by the latter strain, but inducibly by the former.

The reason why both ethanolamine and a cobalamin, acting in concert, are required to induce ammonia-lyase formation, is uncertain. Experiments using chloramphenicol previously showed that enzyme synthesis *de novo* was involved (Blackwell et al., 1977). The evidence available suggests that, according to the lac operon model for enzyme regulation (Jacob & Monod, 1961), the binding of repressor protein to the operator region for the DNA sequence specifying the apoenzyme has its affinity modified by ethanolamine and cobalamin acting together but not separately. The constitutive mutants described above would thus seem to have suffered mutation affecting the regulator or operator genes for the operon. The validity of this hypothesis, whether or not the repressor protein possesses separate binding sites for the co-inducers and indeed whether the lac operon model is appropriate, must await further genetic studies. The term 'concerted' induction has been proposed for the phenomenon (Blackwell et al., 1977), 'multivalent' having mechanistic implications. The term 'multivalent induction' has been applied, however, to the induced formation of catabolic L-threonine hydro-lyase (deaminating) in *E. coli* by the concerted action of threonine and three other amino acids (Yui et al., 1977).

The general metabolic significance of ethanolamine ammonia-lyase, i.e. playing a catabolic role, was underlined by the fact that it was subject to catabolite repression. In contrast, enzyme formation was unaffected by alternative sources of nitrogen present during growth (Scarlett & Turner, 1976). All adenosylcobalamin-requiring enzymes found in bacteria catalyse steps in catabolic pathways (Stadtman, 1971; Babior, 1975; Krouwer & Babior, 1977). Ethanolamine ammonia-lyase formation was repressed by preferred growth substrates, particularly glucose and other sugars in *E. coli* wild-type (Blackwell et al., 1977), also in the mutant strains where enzyme formation was constitutive. This finding was consistent with the promoter region of the operon (Beckwith, 1964; Jacob & Monod, 1965) being unaffected by the mutations.

Although ethanolamine ammonia-lyase is known to be a substrate-induced enzyme in *Clostridium*
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(Bradbeer, 1965), no inducer role for a cobamide has been reported. In contrast with E. coli, which is known to be incapable of synthesis of vitamin B₁₂ de novo (Foster et al., 1964), many clostridia are known to synthesize corrinoid compounds. Thus C. sticklandii synthesizes large amounts of B₁₂ compounds, particularly coenzyme B₁₂, and exhibits at least seven coenzyme B₁₂-dependent enzyme-catalysed reactions (see Stadtman, 1971). The possibility exists that some endogenously synthesized cobamide is involved in ethanolamine ammonia-lyase induction in clostridia as well as enterobacteria.

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