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CRYSTALLIZATION NOTES

Crystallization and Preliminary X-ray Diffraction Studies of the Cobalamin-binding Domain of Methionine Synthase from *Escherichia coli*

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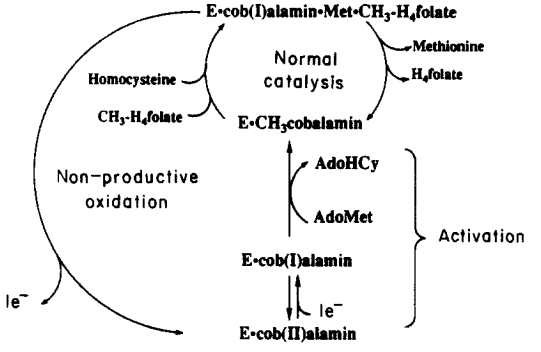
Crystals of a cobalamin-binding domain (M_r = 28,000) have been grown in polyethylene glycol 6000 at pH 7·5, starting from solutions of intact (M_r = 133,000) cobalamin-dependent methionine synthase. The crystals are orthorhombic in space group $P2_12_12_1$, with cell dimensions a = 96·9 Å, b = 55·4 Å, c = 103·8 Å. For two molecules per asymmetric unit, the calculated V_M value is 2·45 Å^3/Da. A native data set has been collected to 3 Å resolution.

Keywords: crystallization; methionine synthase; 5-methyltetrahydrofolate-homocysteine methyl transferase (E.C. 2.1.1.13); cobalamin; X-ray diffraction

Methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase; E.C. 2.1.1.13) catalyzes the transfer of a methyl group from methyltetrahydrofolate to homocysteine, generating tetrahydrofolate and methionine. In prokaryotes, this methyl transfer is the final step in methionine biosynthesis; in mammals, which do not synthesize methionine *de novo*, methionine synthase regenerates methionine from homocysteine to maintain the pool of *S*-adenosylmethionine (AdoMet‡). Inhibition of human methionine synthase has been associated with the symptoms of vitamin B_{12} deficiency (including the development of megaloblastic anemia), and results in diminished intracellular levels of folate. Because inhibition of this enzyme limits the availability of folate for nucleotide biosynthesis, methionine synthase is a potential chemotherapeutic target (Banerjee & Matthews, 1990).

The function and properties of cobalamin-dependent methionine synthase have been the subject of a review (Banerjee & Matthews, 1990). Methionine synthase differs from other cobalamin-dependent enzymes in several significant ways. Catalysis proceeds *via* a double displacement mechanism involving methylcobalamin and cob(I)alamin as intermediates (Scheme 1: and see Banerjee *et al.*, 1990), rather than *via* radical intermediates. The presence of the methylcobalamin intermediate distinguishes methionine synthase from the cobalamin-dependent mutases, in which the ligand to cobalt is the 5′ methylene carbon atom of a deoxyadenosyl moiety.



Scheme 1. Postulated reaction mechanism of methionine synthase showing the interconversion of the various oxidation states of E · cobalamin. While cob(III)alamin is readily reduced to cob(II)alamin, the reduction of cob(II)alamin to cob(I)alamin is thermodynamically unfavorable and must be coupled to an exergonic methyl transfer using AdoMet as the methyl donor.

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‡ Abbreviations used: AdoMet, *S*-adenosylmethionine; PEG, polyethylene glycol; TLCK, N^{α}-p-tosyl-L-lysine chloromethyl ketone.

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Cobalamin-dependent enzymes tend to be large, complex molecules, with multidomain and/or multisubunit structures and, as a result, are difficult subjects for X-ray analysis. No structure of a cobalamin-dependent enzyme is presently available, although crystallization of the adenosylcobalamin-dependent methylmalonyl-CoA mutase from *Propionibacterium shermanii* has been reported (Marsh *et al.*, 1988; McKie *et al.*, 1990). Methionine synthase is a single but very long polypeptide of $M_r = 133{,}000$ (Frasca *et al.*, 1988; Old *et al.*, 1990). Here, we report the crystallization of a 28 kDa cobalamin-binding fragment of methionine synthase. Cleavage of the holoprotein occurs *in situ* in crystallization experiments using PEG 6000 as precipitant, possibly as a result of protease contamination. The cobalamin-binding fragment that crystallizes appears to be a stable domain and is tractable for structure determination.

Purification and stability of the holoprotein

The purification of methionine synthase from a strain of *Escherichia coli* that overexpresses the enzyme has been described (Banerjee *et al.*, 1989). In the preparations used for crystallization experiments, chromatography on hydroxyapatite (Bio-Gel HTP: Frasca *et al.*, 1988), was reintroduced to remove minor impurities. The purified protein ran as a single band on SDS/polyacrylamide gel electrophoresis. It should be noted that serine protease inhibitors, TLCK and phenylmethylsulfonyl fluoride, are added at two different stages of the purification. During a period of several days at room temperature, solutions of purified holoprotein are gradually degraded, yielding after several weeks a series of fragments similar to those shown in lane 2 of Figure 1. This behavior is consistent with the presence of a protease that co-purifies with the holoprotein, but other cleavage mechanisms cannot be excluded.

Reductive methylation of the cobalamin in methionine synthase

The purified protein was found by spectral analysis to be a mixture of species: cob(II)alamin, cob(III)-alamin and methylcobalamin. The cob(II)alamin and the cob(III)alamin forms are catalytically inactive and must be activated by reductive methylation (see Scheme 1). For crystallization experiments, we preferred to start with a single species. The methylcobalamin form is relatively stable, is an intermediate in the methyl transfer pathway, and is readily obtained from the cob(II) or cob(III) species by reductive methylation. The cob(II)alamin and cob(III)alamin forms were therefore converted to methylcobalamin by an electrochemical technique as described (Banerjee *et al.*, 1990). An enzyme solution that was 1 mM in AdoMet and 500 μM in methyl viologen was poised at -450 mV *versus* the standard hydrogen electrode for 30 minutes under an atmosphere of argon gas in an electrochemical cell (Harder *et al.*, 1989) to give complete conversion of the enzyme-bound cofactor to methylcobalamin. The cell was then opened to the atmosphere and the phosphate buffer was replaced with crystallization buffer (50 mM-Tris · HCl, pH 7·2) by repeated concentration of the enzyme solution in a Centricon 30 microconcentrator (Amicon) at 4°C.

Methylcobalamin in solution readily decomposes to aquocob(III)alamin in a light and oxygen catalyzed reaction, but loss of the methyl group is retarded when the cofactor is bound to methionine synthase. In order to preserve cofactor homogeneity, manipulations subsequent to methylation were carried out with minimal exposure to light and the crystallization trays were wrapped in foil. The methylated enzyme is stored in portions at -80°C.

Crystallization of the cobalamin-binding domain

Crystals were grown in hanging drops (McPherson, 1982), at room temperature, using PEG 6000 as precipitant. The hanging drops were set up with holoprotein, $M_r = 133{,}000$, at 5 to 10 mg/ml concentrations, 6 to 8% PEG 6000 (Fluka) in 50 mM-Tris · HCl, pH 7·5, 4·5 mM-sodium azide and 5 mM-cadmium chloride, and were equilibrated against 15% PEG 6000 in 50 mM-Tris buffer. Drop volumes varied from 4 to 8 μl. The crystals take more than a month to grow. During this time, the holoprotein is cleaved in the hanging drop, yielding a mixture of fragments. By gel electrophoresis, we have determined that the 28 kDa fragment is selectively incorporated in the crystals (Fig. 1). Although this phenomenon is unusual, it appears to be entirely reproducible. All six of the protein preparations tested have given the same results, and approximately 70% of the trays set up as described above have produced crystals suitable for data collection. It is interesting to note

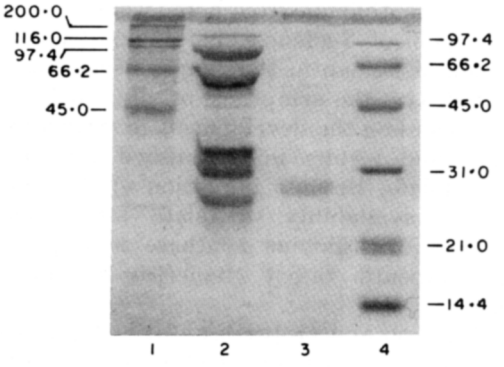

Figure 1. Electrophoretic analysis of redissolved crystals (lane 3) and contents of the hanging drop after crystals had formed (lane 2). A small amount of holoprotein (133 kDa) appears to be left in the hanging drop (top band lane 2). Most of the protein, however, has been cleaved into fragments that range in size from approximately 25 to 100 kDa. In contrast, redissolved crystals produce a single, clean band (lane 3) that corresponds to M_r 28,000. Molecular mass markers are shown in lanes 1 and 4.

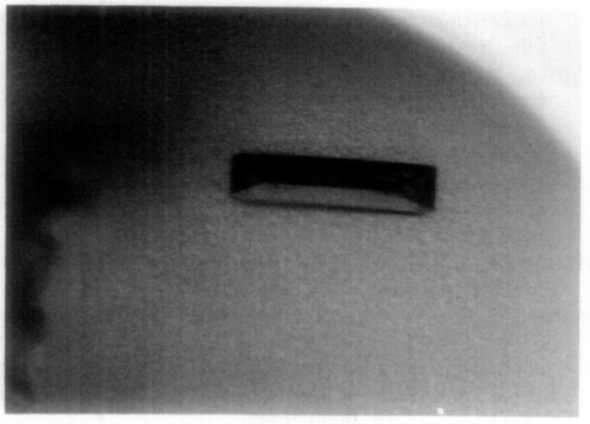

Figure 2. A crystal of the 28 kDa fragment of methionine synthase. The red color indicates the presence of bound cobalamin. Crystal dimensions 0·54 mm × 0·18 mm.

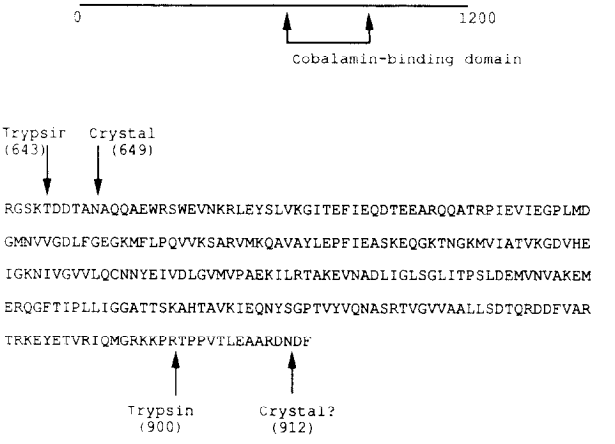

Figure 3. The location of the cobalamin-binding fragments of methionine synthase. As shown in the upper diagram, the cobalamin-binding domain starts approximately halfway through the sequence of the intact protein. The N terminus and the proposed C terminus of the protein that crystallizes are as indicated. Digestion with trypsin yields a related cobalamin-binding fragment spanning residues 643 through 900.

that crystals of the 28 kDa fragment have been obtained only at room temperature (17 to 24 °C). Cleavage of the holoprotein is limited at 4 °C; gel analysis of a droplet stored for about two years at 4 °C showed predominantly intact protein.

Partial characterization of the cobalamin-binding domain

The red color of the crystals identifies the 28 kDa fragment as the cobalamin-binding domain (Fig. 2). The N-terminal sequence of protein from the crystal is A-Q-Q-A, establishing that the intact protein is cleaved between Asn648 and Ala649, approximately halfway through the sequence. We have attempted to determine the C-terminal sequence by analysis of a lysyl endopeptidase (LysC) digest of the protein from redissolved crystals. Amino acid compositions of the LysC fragments were consistent with the sequence shown in Figure 3. The C-terminal fragment, which was identified by the absence of lysine, had a high proline content, consistent with the segment starting with Pro899 (Fig. 3). It was not possible to determine the exact length or composition of the peptide because of low yield; however the C terminus appears to extend 9 to 13 residues beyond Pro899, suggesting that the C terminus of the peptide is Asn912. Attempts to clone and express the fragment Ala649–Asn912 are in progress, beginning with amplification of the corresponding DNA sequence by polymerase chain reaction.

The region of methionine synthase depicted in Figure 3 had been associated with cobalamin binding: trypsin cleaves the intact protein six residues upstream from Asn648 and again after Arg900 to yield a 28 kDa fragment that contains bound cobalamin (Banerjee *et al.*, 1989). Cleavage at Arg900 is accompanied by a loss of enzyme activity in the standard assay. We have not been able to purify the tryptic cleavage product for characterization or crystallization, because it is only a minor product of proteolysis with trypsin (Banerjee *et al.*, 1989). Initial experiments reveal that critical binding determinants for AdoMet, which is required for activation by reductive methylation (Scheme 1), lie in the 37 kDa domain downstream from the cobalamin-binding domain (R. M. Blumenthal & R.G.M., personal communication).

Characterization of crystals of the cobalamin-binding domain

The crystals diffract to at least 2·7 Å (1 Å = 0·1 nm). Characterization of the crystals, both at the Cornell High Energy Synchrotron Source (CHESS) and with a Xuong–Hamlin multiwire area detector system (Hamlin, 1985; Howard *et al.*, 1985), showed *mmm* diffraction symmetry with cell dimensions $a = 96{\cdot}9$ Å, $b = 55{\cdot}4$ Å, $c = 103{\cdot}8$ Å. From the systematic absences, the space group was determined to be $P2_12_12_1$. The Matthews coefficient, V_M, corresponding to two molecules per asymmetric unit (2·45 Å^3/Dalton) falls into the commonly observed range (Matthews, 1968), whereas the value for one molecule per asymmetric unit (4·9 Å^3/Dalton) does not. The solvent content is approximately 52% (v/v), using a value of 0·75 cm^3/g for the protein specific volume and assuming two molecules per asymmetric unit.

A native data set was collected to 3 Å resolution with a Xuong–Hamlin multiwire area detector system using a Rigaku RU200 rotating anode X-ray generator as the X-ray source. The beam emerging from a graphite monochromator (Supper) was collimated to a diameter of 0·3 mm. Crystals last in the beam (100 mA, 50 mV) for approximately 70 hours without significant decay, making it possible to collect a complete data set on one crystal. The data

set has threefold redundancy with 10,589 of the 11,677 possible unique reflections measured to 3·0 Å. The overall merging R value is 0·054

$$(R = \Sigma_{hkl}\sum_i |I_i - \langle I \rangle| / \sum \langle I \rangle).$$

Crystallization of the cobalamin-binding domain from methionine synthase affords a unique opportunity to study the protein–ligand interactions that determine how vitamin B_{12} is bound by methionine synthase, and may provide more general insight into how proteins bind corrins. The binding domain described here may also provide the first structural insights into how methionine synthase modulates the reactivity of the carbon–cobalt bond, providing a valuable complement to mechanistic analysis.

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References

Banerjee, R. V. & Matthews, R. G. (1990). Cobalamin-dependent methionine synthase. *FASEB J.* **4**, 1450–1459.

Banerjee, R. V., Johnston, N. L., Sobeski, J. K., Datta, P. & Matthews, R. G. (1989). Cloning and sequence analysis of the *Escherichia coli metH* gene encoding cobalamin-dependent methionine synthase and isolation of a tryptic fragment containing the cobalamin-binding domain *J. Biol. Chem.* **264**, 13888–13895.

Banerjee, R. V., Frasca, V., Ballou, D. P. & Matthews, R. G. (1990). Participation of cob(I)alamin in the reaction catalyzed by methionine synthase from *Escherichia coli*: A steady-state and rapid reaction kinetic analysis. *Biochemistry*, **29**, 11101–11109.

Frasca, V., Banerjee, R. V., Dunham, W. R., Sands, R. H. & Matthews, R. G. (1988). Cobalamin-dependent methionine synthase from *Escherichia coli* B: Electron paramagnetic resonance spectra of the inactive form and the active methylated form of the enzyme. *Biochemistry*, **27**, 8458–8465.

Hamlin, R. (1985). Multiwire area X-ray diffractometers. *Methods Enzymol.* **114**, 416–452.

Harder, S. R., Wei-Ping, L., Feinberg, B. A. & Ragsdale, S. W. (1989). Spectroelectrochemical studies of the corrinoid/iron-sulfur protein involved in acetyl coenzyme A synthesis by *Clostridium thermoaceticum*. *Biochemistry*, **28**, 9080–9087.

Howard, A. J., Nielsen, C. & Xuong, Ng. H. (1985). Software for a diffractometer with multiwire area detector. *Methods Enzymol.* **114**, 452–472.

Marsh, N., Leadlay, P. F. & Evans, P. R. (1988). Crystallization and preliminary diffraction data for adenosylcobalamin-dependent methylmalonyl-CoA mutase from *Propionibacterium shermanii*. *J. Mol. Biol.* **200**, 421–422.

Matthews, B. (1968). Solvent content of protein crystals. *J. Mol. Biol.* **33**, 491–497.

McKie, N., Keep, N. H., Patchett, M. L. & Leadlay, P. F. (1990). Adenosylcobalamin-dependent methylmalonyl-CoA mutase from *Propionibacterium shermanii*. *Biochem. J.* **269**, 293–298.

McPherson, A. (1982). *Preparation and Analysis of Protein Crystals*, pp. 82–159. Wiley and Sons, New York.

Old, I. G., Margarita, D., Glass, R. E. & Saint Girons, I. (1990). Nucleotide sequence of the *metH* gene of *Escherichia coli* K-12 and comparison with that of *Salmonella typhimurium* LT2. *Gene*, **87**, 15–21.

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