Adenosylcobalamin-dependent methylmalonyl-CoA mutase from *Propionibacterium shermanii*

Active holoenzyme produced from *Escherichia coli*

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The linked structural genes coding for both subunits of adenosylcobalamin-dependent methylmalonyl-CoA mutase from the Gram-positive bacterium *Propionibacterium shermanii* have been altered by site-directed mutagenesis and placed under the control of an inducible phage-T7-specific plasmid promoter in *Escherichia coli*. Conditions have been found under which both α - and β -subunits are produced in soluble form, in near 1:1 ratio, and assemble to form apo-mutase totalling about 5% of the total cellular protein. Methylmalonyl-CoA mutase purified from these cells could be readily converted into the holoenzyme by addition of adenosylcobalamin. The active holoenzyme apparently crystallizes in the same space group as an inactive corrinoid-containing form of the enzyme obtained previously.

INTRODUCTION

Methylmalonyl-CoA mutase (EC 5.4.99.2) is an adenosylcobalamin-dependent enzyme that catalyses the structural rearrangement of succinyl-CoA (3-carboxypropionyl-CoA) into (2*R*)-methylmalonyl-CoA as part of the propionic acid fermentation pathway in *Propionibacterium shermanii* (Overath *et al.*, 1962; Kellermeyer *et al.*, 1964). Reactions that require adenosylcobalamin (coenzyme B₁₂) have been intensively studied [for reviews, see Babior & Krouwer (1979), Dolphin (1982) and Golding & Rao (1986)], and the first step in the catalysis is widely agreed to involve homolytic cleavage of the unusual carbon–cobalt bond in the cofactor (Abeles, 1979; Finke & Hay, 1984; Halpern, 1985). Further progress in defining the mechanistic pathway of these enigmatic rearrangements will require detailed information about the structure and active-site chemistry of adenosylcobalamin-dependent enzymes.

Recently, the structural genes for methylmalonyl-CoA mutase have been cloned from *Propionibacterium shermanii* (Leadlay *et al.*, 1989; Marsh *et al.*, 1989a) and from a human cell line (Ledley *et al.*, 1988; Jansen *et al.*, 1989). In contrast with the human enzyme, which is isolated as a homodimer, the mutase from *P. shermanii* contains two homologous, but non-identical, subunits (Francalanci *et al.*, 1986; Woelfle *et al.*, 1986). Comparison of the protein sequence inferred from the nucleotide sequence of these genes has revealed that the human mutase is strikingly similar to the α -subunit (*M_r* 80 100), but far less similar to the β -subunit (*M_r* 69 500) of the enzyme from *P. shermanii* (Leadlay & Ledley, 1989). A corrinoid-containing inactive form of the mutase from *P. shermanii* has been crystallized in a form suitable for high-resolution X-ray crystallography (Marsh *et al.*, 1988), but it would clearly be preferable to carry out structural analysis on the active holoenzyme itself. To allow the preparation of large quantities of either apo- or holo-mutase, we have therefore sought to express the mutase genes from *P. shermanii* in *Escherichia coli*.

Genes from *P. shermanii* are expressed poorly, if at all, in *E. coli* from their own promoters (N. K. Davis, N. McKie & P. F. Leadlay, unpublished work; Murtif *et al.*, 1985). Here we report the engineering of the linked mutase genes and their efficient

expression from a powerful plasmid promoter in *E. coli*, and the subsequent purification of methylmalonyl-CoA mutase from extracts of the recombinant *E. coli* cells. Active homogeneous holoenzyme has been reconstituted from this material and crystallized. The preparation of mutant enzymes by site-directed mutagenesis can also be conveniently carried out with this system.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli TG1 (Biggin *et al.*, 1983) was kindly given by Dr. E. S. Ward, MRC Laboratory for Molecular Biology, Cambridge, U.K., and *E. coli* TG1 *recO1504::Tn5* (Kolodner *et al.*, 1985), a recombination-deficient mutant derived from TG1, was kindly given by Dr. P. Oliver, Department of Genetics, University of Cambridge, Cambridge, U.K. Both strains were maintained on M9 minimal medium supplemented with 0.1 mM-thiamin hydrochloride (Maniatis *et al.*, 1982) to maintain selection for the F' episome. The *E. coli* K38 strain used for expression of methylmalonyl-CoA mutase has been described (Russel & Model, 1984). *E. coli* strains were grown at 37 °C in 2 × TY broth [1% yeast extract/1% tryptone (Difco Laboratories, Detroit, MI, U.S.A.)/0.5% NaCl] unless otherwise noted. *E. coli* plasmid DNA was isolated by using the alkaline-lysis procedure of Kieser (1984).

DNA manipulations

Procedures for isolation of DNA restriction fragments from agarose gels, DNA ligation and the transformation of *E. coli*, and for purification by PAGE and phosphorylation of oligonucleotides used for site-directed mutagenesis, either have been described previously (Marsh *et al.*, 1989a) or were performed by using standard methods (Maniatis *et al.*, 1982).

DNA sequencing and analysis

DNA was sequenced by using the dideoxy-chain-termination method (Sanger *et al.*, 1977), with either reverse primer or specific oligonucleotide primers. Oligonucleotides were syn-

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thesized in a Biosearch Cyclone DNA Synthesiser, and were purified before use by preparative electrophoresis in polyacrylamide gels (Ogden & Adams, 1987). Sequencing was carried out with modified phage-T7 DNA polymerase (Sequenase) (Tabor & Richardson, 1987), essentially according to the manufacturer's instructions (Zhang *et al.*, 1988). RNA and oligonucleotide sequences were screened for potential stem-loop structures using the program ANALYSEQ (Staden, 1984), and potential alternative binding sites for mutagenic oligonucleotides were sought using the FIND program (Devereux *et al.*, 1984).

Subcloning of DNA fragments containing the linked structural genes for methylmalonyl-CoA mutase

A 4.3 kbp *Sma*I fragment containing both mutase genes was excised from plasmid pMK1 (Marsh *et al.*, 1989a), isolated by elution from an agarose gel, and ligated with *Sma*I-cut and dephosphorylated phagemid vector pTZ18R (Pharmacia). The ligation mixture was used to transform *E. coli* TG1 *recO1504::Tn5*. Recombinants were selected on 2 × TY agar plates supplemented with ampicillin (100 µg/ml), 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside (25 µg/ml) and isopropyl β-D-thiogalactopyranoside (20 µg/ml). One recombinant (pM7), containing an *Sma*I insert with the mutase genes in the correct orientation for transcription from the vector-borne phage-T7 promoter, was selected for further work.

Construction and use of pMEX1 and pMEX2 expression vectors

An *Nde*I site spanning the initiation codon on the *P. shermanii* *mutA* gene (encoding the β-subunit of methylmalonyl-CoA mutase) was introduced by oligonucleotide-directed mutagenesis (Zoller & Smith, 1982) of a single-stranded DNA template produced from pM7 under helper-phage infection, using the 'in vitro' mutagenesis kit (Nakamaye & Eckstein, 1986) supplied by Amersham International (Amersham, Bucks., U.K.). To create the *Nde*I site, a two-base substitution was introduced by using the mutagenic primer 5'-CAGGAGTCATATGAGCAGC-3' (the change is shown in bold face). The resulting plasmid was designated 'pM7mut3'. After confirmation of the specific alteration, the 4.2 kbp *Nde*I-*Hind*III fragment was excised from pM7mut3 and ligated into pT7-7 (Tabor & Richardson, 1985; S. Tabor, unpublished work), an expression vector containing a phage-T7 RNA polymerase promoter, to give the expression plasmid pMEX1 (see the Results section and Fig. 1). An additional alteration, in which the start codon for the *mutB* gene was changed from GTG to ATG, was carried out in a similar way: a single-base substitution was introduced into pM7mut3 by using the mutagenic primer 5'-GGGAGTCGCGAA-ATGAGCACT-3'. One of the recombinant clones obtained,

designated 'pM7mut3A', was sequenced to confirm this second alteration, and the 4.2 kbp *Nde*I-*Hind*III fragment containing the mutase genes was subcloned into pT7-7 to create the expression plasmid pMEX2 (see the Results section and Fig. 1). These expression plasmids were transformed into the host strain for expression, namely *E. coli* K38 containing pGP1-2. The pGP1-2 plasmid carries the gene for phage-T7 RNA polymerase under the control of a temperature-sensitive repressor (Tabor & Richardson, 1985). For initial experiments on the expression of the mutase genes from pMEX1, *E. coli* K38 (pGP1-2, pMEX1) cells were grown at 30 °C overnight in 2 × TY medium supplemented with ampicillin (100 µg/ml) and kanamycin (75 µg/ml) and then diluted 100-fold in fresh ampicillin- and kanamycin-supplemented 2 × TY medium. When the A_{600} of this culture had reached 0.6, the cells were subjected to heat-shock at 42 °C for 30 min, to induce methylmalonyl-CoA mutase, and incubated at 37 °C for a further 2 h. The mutase genes were initially expressed from pMEX2 using an analogous procedure. Protein samples, obtained by resuspending and boiling cell pellets in SDS/PAGE sample buffer for 5 min, were analysed on SDS/15% polyacrylamide gels, cross-linked with 0.1% bisacrylamide, using the discontinuous buffer system of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue R250.

Enzyme and protein assays

Methylmalonyl-CoA mutase was assayed at 30 °C, with succinyl-CoA as substrate, essentially by the method of Zagalak *et al.* (1974). Protein concentrations were determined by the method of Bradford (1976), with BSA as the standard.

N-Terminal sequence analysis

The individual subunits of methylmalonyl-CoA mutase were electro-eluted from an SDS/polyacrylamide gel on to a poly(vinylidene difluoride) membrane (Matsudaira, 1987), and the N-terminal amino acid sequence (ten residues) of each subunit was determined by using an Applied Biosystems model-477A pulsed-liquid sequencer, fitted with an on-line model 120A detector for measurement of amino acid phenylthiohydantoin derivatives.

Purification of methylmalonyl-CoA mutase from recombinant *E. coli*

Growth and induction. The expressing strain was picked as a single colony from 2 × TY plates containing ampicillin (100 µg/ml) and kanamycin (75 µg/ml). This was grown overnight in 4 ml of M1 medium containing the same concentrations of antibiotics. M1 medium contains, per litre: 15 g of casamino acids (Difco), 5 g of yeast extract, 10 ml of 0.4 M-sodium phosphate buffer, pH 7.2, 10 ml of solution A and 1 ml of solution B, adjusted to pH 7.2 with 5 M-NaOH. The composition of solution A is 1 M-NH₄Cl, 50 mM-K₂SO₄, 50 mM-MgSO₄ and 2 mM-CaCl₂, and the composition of solution B is 0.1 M-HCl, 10 mM-FeSO₄, 2 mM-MnCl₂, 2 mM-ZnSO₄, 0.2 mM-CoSO₄, 0.1 mM-CuSO₄ and 0.1 mM-NiCl₂. The overnight culture was used to inoculate five baffled 2-litre flasks, each with 600 ml of M1 medium, containing ampicillin (100 µg/ml) and kanamycin (75 µg/ml). The cultures were grown at 28 °C until the A_{600} reached 1.75. Then, 400 ml of M1 medium, preheated to 65 °C, was added to each culture and the flask was shaken vigorously for 1 min and then cooled to 28 °C by shaking in iced water for 1 min. The cultures were allowed to grow for an additional 2.5–3 h at 28 °C and were then harvested by centrifugation.

Initial extraction. *E. coli* cell paste (20 g) was suspended in 200 ml of 0.1 M-Tris/HCl (pH 7.5)/5 mM-EDTA/5 mM-benzamidine hydrochloride / 2 mM - dithiothreitol / 0.5 mM - phenylmethane-

Fig. 1. Plasmid map of the expression plasmid pMEX1

MutA and *MutB* encode the β- and α-subunits of methylmalonyl-CoA mutase respectively. The *bla* gene encodes β-lactamase. Abbreviations: RBS, ribosome-binding site; T7, phage T7.

sulphonyl fluoride. The cells were disrupted either by two passes through an automated French press (Aminco) operated at 7.5 MPa (1100 lbf/in²) or by sonication using an ultrasonic processor (model W-225; Heat Systems-Ultrasonics, Farmingdale, NY, U.S.A.) operated at full power in eight 2 min bursts with 5 min cooling intervals. The mixture was centrifuged (12000 *g* at 4 °C for 30 min) to obtain the crude extract.

Q-Sepharose chromatography. The extract was diluted with an equal volume of water and loaded directly on to a Q-Sepharose (fast flow) column (Pharmacia; 5 cm × 20 cm) previously equilibrated with buffer N (50 mM-Tris/HCl, pH 7.5, containing 0.2 mM-EDTA, 1 mM-benzamidine hydrochloride, 2 mM-dithiothreitol and 0.1 mM-phenylmethanesulphonyl fluoride). After washing with buffer N the enzyme was eluted with a 1-litre linear 0–0.6 M-KCl gradient in buffer N at a flow rate of 150 ml/h. Active fractions were eluted towards the end of the gradient, and were pooled and dialysed against the buffer N.

Anion-exchange f.p.l.c. A Pharmacia Mono Q (10/10) column and Pharmacia f.p.l.c. (GP-250 gradient programmer and P-500 pumps) were used. The running buffers were buffer N and 1 M-Tris/HCl, pH 7.5, containing the same added ingredients as buffer N. Half of the active material from the previous step was loaded on to the column in a single run. Proteins were eluted, at a flow rate of 2 ml/min, by using a linear gradient from 0 to 0.4 M-Tris/HCl over 100 min. The largest protein peak to emerge was methylmalonyl-CoA mutase, approx. 95% homogeneous as judged by SDS/PAGE analysis (Laemmli, 1970). The pooled active fractions were dialysed against buffer N, and adenosylcobalamin was added to a final concentration of 50 µM. The material was loaded on to the Mono Q column in two batches and eluted as described above. The apoenzyme emerged just ahead of the holoenzyme, near the end of the buffer gradient.

Crystallization of the methylmalonyl-CoA mutase holoenzyme

The holoenzyme was concentrated to 20 mg/ml by using a Centriprep 30 unit (Amicon), and crystallization trials were set up using the hanging-drop technique, with poly(ethylene glycol) as the precipitant, as described by Marsh *et al.* (1988). Crystals were obtained from a solution of 24% (w/v) poly(ethylene glycol) 8000 in 50-mM-Tris/HCl, pH 7.5, containing 5 mM-EDTA, 2 mM-CoASH, 2 mM-dithiothreitol and 1 mM-benzamidine hydrochloride.

RESULTS

High-level expression in *E. coli* of the genes for *P. shermanii* methylmalonyl-CoA mutase

When the *P. shermanii* structural genes were subcloned into various high-copy-number plasmid vectors in *E. coli*, only trace amounts of mutase polypeptides could be detected after Western blotting of cell-free extracts from these recombinants (N. McKie, unpublished work), and therefore a more efficient expression system was sought. The strategy finally adopted (outlined in Fig. 1) was one that has proved in our hands to be successful for other genes from Gram-positive bacteria (see, for example, Swan *et al.*, 1989); the removal of all 5'-non-coding sequence and positioning of the genes behind a phage-T7-specific promoter (Tabor & Richardson, 1985; S. Tabor, unpublished work). The linked mutase genes were excised from plasmid pMK1 and subcloned together on a 4.3 kbp *Sma*I fragment, into the phagemid vector pTZ18R. Instability of the *Sma*I fragment was noted after subcloning in various *E. coli* vectors. Fortunately, this instability was overcome by using a derivative of *E. coli* strain TG1 deficient in recombination (TG1 *recO*) as the host for the subcloning in

Fig. 2. Expression of methylmalonyl-CoA mutase in *E. coli* K38 (pGP1-2) from pMEX1 and pMEX2 under two heat-shock regimes

Cultures were grown at 30 °C before heat-shock induction and growth was continued for 2 h at the end of the heat-shock period. Whole-cell protein extracts were prepared from cell pellets. Molecular-mass markers ('MW-SDS-200') were from Sigma. a, 2 µg of methylmalonyl-CoA mutase purified from *P. shermanii*; b, pMEX1 before heat-shock induction; c, pMEX2 before heat-shock induction; d, pMEX1, 1 min heat-shock at 42 °C, followed by growth at 28 °C; e, pMEX2, 1 min heat-shock at 42 °C, followed by growth at 28 °C; f, pMEX1, 30 min heat-shock at 42 °C, followed by growth at 37 °C; g, pMEX2, 30 min heat-shock at 42 °C, followed by growth at 37 °C; h, *E. coli* K38 (pGP1-2, pT7-7), 30 min heat-shock at 42 °C, followed by growth at 37 °C.

pTZ18R. Single-stranded phages were obtained from pTZ18R recombinants after infection with helper phage VCSM13 (Stratagene, San Diego, CA, U.S.A.) and single-stranded DNA purified from these phages was used as the template in oligonucleotide-directed mutagenesis (Nakamaye & Eckstein, 1986) to introduce an *Nde*I site at the start codon of *mutA*. Subsequently, *Nde*I–*Hind*III fragments were subcloned into the expression plasmid pT7-7, and the recombinants, pMEX1 and pMEX2, were used to transform *E. coli* K38 containing pGP1-2, a plasmid housing the gene for phage-T7 RNA polymerase under the control of a temperature-sensitive repressor (Tabor & Richardson, 1985). The mutase expression plasmids pMEX1 and pMEX2 differ only in that pMEX2 has the GTG initiation codon for the downstream gene (*mutB*) replaced by ATG. The TGA stop codon for the *mutA* gene overlaps the GTG start codon for the *mutB* gene and this change alters the final codon of *mutA* from AAG to AAA, but this does not alter the amino acid specified (lysine).

The mutase genes were induced by various heat-shock protocols (see the Materials and methods section and Fig. 2) and the proteins in the *E. coli* cells were analysed by SDS/PAGE (Fig. 2). The results showed that, after induction, the mutase polypeptides constituted a significant proportion (up to 35%) of total cell protein in the recombinant *E. coli*. However, at the very high levels of expression obtained by using a 30 min heat-shock, the β-subunit, the product of the upstream *mutA* gene, was being produced in considerable excess. Scanning densitometry showed that the β/α-subunit ratio was 4.6:1 for pMEX1 and 3.5:1 for pMEX2, a considerable departure from the desired subunit ratio of 1:1. A further drawback was that essentially all of the mutase polypeptides produced under these conditions were obtained in the form of inclusion bodies (Marston, 1986).

Fig. 3. Effect of the duration of heat-shock induction on the solubility of methylmalonyl-CoA mutase polypeptides expressed in *E. coli*

Cultures of *E. coli* K38 (pGP1-2, pMEX2) were grown at 28 °C before heat-shock and harvested by centrifugation. Cell pellets were resuspended in 0.1 M-Tris/HCl, pH 8, containing 2 mM-EDTA, 2 mM-dithiothreitol, 10 mM-benzamidinium hydrochloride and 0.5 mM-phenylmethanesulphonyl fluoride, and disrupted completely by sonication on ice. The cell lysate was centrifuged for 1 h at 4 °C and 150 000 *g* (r_{av} , 8.35 cm) and the pellet of cell debris (insoluble fraction) was resuspended in sonication buffer. a and b, heat-shocked for 1 min at 42 °C, followed by 3 h growth at 28 °C (a, insoluble fraction; b, soluble fraction); c and d, heat-shocked for 30 min at 42 °C, followed by 2 h growth at 37 °C (c, insoluble fraction; d, soluble fraction); e, 2 µg of methylmalonyl-CoA mutase purified from *P. shermanii*.

Further experiments showed that the conditions of induction could be altered so as to produce the methylmalonyl-CoA mutase in soluble form. As shown in Fig. 3, a shorter heat-shock followed by incubation at 28 °C was effective in preventing the formation of inclusion bodies. This finding is consistent with the results of other workers (Schein & Noteborn, 1988; Haase-Pettingell & King, 1988) and may be of wider significance in the expression of heterologous proteins in *E. coli* using heat-shock. In addition, the subunit ratio was close to 1:1, which is expected to favour the correct folding of the $\alpha\beta$ heterodimer. Under these conditions, correctly folded mutase represented about 5% of total cell protein after induction.

Purification and crystallization of methylmalonyl-CoA mutase

Methylmalonyl-CoA mutase was purified from recombinant *E. coli* cells containing plasmid pMEX2 by a modification of the procedure used for the enzyme produced in *P. shermanii* (Francalanci *et al.*, 1986). The use of $(\text{NH}_4)_2\text{SO}_4$ precipitation, which has been shown to promote the dissociation of the enzyme into its subunits (Marsh *et al.*, 1989b), was avoided. Column chromatography on Q-Sepharose (fast flow) gave better yields of active mutase than did columns of DEAE-cellulose. As a final step, the apoenzyme was converted into the holoenzyme by the addition of adenosylcobalamin, and the holoenzyme was separated from remaining trace impurities and from the apoenzyme by a second chromatographic run on a Mono Q anion-exchange column. A typical purification is summarized in Table 1. A further 8–10 mg of holoenzyme could be generated by adding coenzyme to the apo-mutase fractions obtained from the second f.p.l.c. step and re-chromatography on the Mono Q column. The

Table 1. Summary of the purification of methylmalonyl-CoA mutase from *E. coli* K38 (pGP1-2, pMEX2)

Sample	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Cell-free supernatant	400	2200	ND*	—
Q-Sepharose (fast flow)	125	612	500	0.8
Mono Q f.p.l.c.				
Without AdoCbl†	33	99	220	2.2
With AdoCbl	21	28	76	2.7

* Not determined; the high level of NADH oxidase activity present in *E. coli* extracts interferes with the assay for methylmalonyl-CoA mutase.

† Abbreviation: AdoCbl, adenosylcobalamin.

subunit sizes, the final β/α ratio and the *N*-terminal sequence of both subunits (*N*-terminal methionine removed) were exactly as for the enzyme produced in *P. shermanii*, and the specific activity of the purified mutase was comparable with that of holo-mutase purified from *P. shermanii* and assayed under the same conditions (N. H. Keep, unpublished results). The holoenzyme was homogeneous, as judged by SDS/PAGE.

The best crystals of holoenzyme obtained so far in hanging-drop trials had dimensions of about 5 mm × 0.1 mm × 0.1 mm, and they diffracted only to about 0.8 nm (8 Å). However, the crystals were apparently in the same space group as those previously obtained with an inactive corrinoid-containing form of mutase (Marsh *et al.*, 1988). The crystals could be separated from the mother liquor and redissolved to give active enzyme, which is particularly encouraging for further work on the X-ray crystal structure of holo-mutase.

DISCUSSION

The recent cloning and sequence analysis of the structural gene for human methylmalonyl-CoA mutase (Ledley *et al.*, 1988; Jansen *et al.*, 1989) and also the genes for methylmalonyl-CoA mutase from *P. shermanii* (Marsh *et al.*, 1989a) have greatly advanced our knowledge of the structure of this adenosylcobalamin-dependent enzyme. There is a strikingly high overall sequence identity between the α -subunit of the *P. shermanii* mutase and the human enzyme, with a rather lower identity between human mutase and the β -subunit of the bacterial enzyme (Leadley & Ledley, 1989). Intriguingly, methylcobalamin-dependent methionine synthase from *E. coli* apparently shares similarities in its *C*-terminal secondary structure with the α -subunit of methylmalonyl-CoA mutase from *P. shermanii* (Banerjee *et al.*, 1989), even though there is no evident sequence similarity.

Knowledge of the gene sequences has now allowed us to develop an efficient system for both site-directed mutagenesis and high-level expression of the *P. shermanii* methylmalonyl-CoA mutase. *E. coli* was chosen as the host organism for this work, principally because of the primitive state of genetic analysis in propionibacteria, but the choice also offered positive benefits: first, since the intracellular cobalamin content of *E. coli* is negligible under aerobic conditions if it is not added to the medium (Jeter *et al.*, 1984, 1989), the mutase should be obtained as the apoenzyme, rather than as the mixture of apo- and cofactor-containing forms found after conventional purification from *P. shermanii*. Secondly, the mutase should be reliably free of the other enzymes of the propionate pathway in *P. shermanii*, particularly methylmalonyl-CoA epimerase, a tenacious trace

contaminant whose presence has bedevilled previous mechanistic analysis of the mutase (Hull *et al.*, 1988).

The TGA stop codon, for the upstream *mutA* gene, encoding the smaller β -subunit of methylmalonyl-CoA mutase from *P. shermanii*, overlaps the GTG start codon for the *mutB* gene, encoding the α -subunit. There is a potential ribosome-binding site appropriately positioned upstream of the *mutB* start codon, within the C-terminal coding sequence of the *mutA* gene, an arrangement that presumably achieves stoichiometric production of subunits (Normark *et al.*, 1983). The imbalance in the expression of the *mutA* and *mutB* genes from the phage-T7 promoter could be partially corrected, at high translation rates, by substitution of the GTG start codon of *mutB* by ATG, which has been noted in other instances to be more efficiently utilized in *E. coli* (Wulff *et al.*, 1984; Reddy *et al.*, 1985; Looman & van Knippenberg, 1986). However, reducing the rate of production of mutase polypeptides (Figs. 2 and 3) was decisive in apparently allowing initiation of translation of *mutB* to keep pace. The formation of inclusion bodies was also avoided under these conditions.

Genes from Gram-positive bacteria (with the notable exception of many *Bacillus* genes) are often poorly expressed in *E. coli*, at least in part because of differences in the structure of the promoter and ribosome-binding site. In this work, a key factor in obtaining efficient translation was the removal of all non-coding DNA upstream of the genes, by introduction of a specific *NdeI* site at the start codon of the *mutA* gene. An integral part of this approach is the use of the phage-T₇-specific plasmid expression vector pT7-7 (S. Tabor, unpublished work), in which a unique *NdeI* site is positioned at an optimal spacing from a ribosome-binding site and the phage-T₇ ϕ 10 promoter. Even before induction, mutase polypeptides were found to be present (Fig. 2).

Comparison of the methylmalonyl-CoA mutase sequences has revealed that no cysteine residues are conserved between the sequences of the human mutase and the α -subunit, and only one cysteine residue is conserved between the human mutase and the β -subunit of the *P. shermanii* enzyme. Several authors have suggested that, during the rearrangements catalysed by adenosylcobalamin-dependent enzymes, an amino acid side chain might participate directly in catalysis as the site of a transient free-radical species (Cleland, 1982; O'Brien *et al.*, 1985; Stubbe, 1988), and interest has centred on the possibility that this species might be a radical derived from a cysteine side chain (Stubbe, 1988, 1989). Even in advance of a high-resolution crystal-structure determination, site-directed mutagenesis can now be used to effect the replacement of individual cysteine residues in methylmalonyl-CoA mutase: an attractively direct way of testing this hypothesis.

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