Carboxymethylation of MutS-Cysteine-15 Specifically Inactivates Adenosylcobalamin-dependent Glutamate Mutase

EXAMINATION OF THE ROLE OF THIS RESIDUE IN COENZYME-BINDING AND CATALYSIS

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The sensitivity of adenosylcobalamin (AdoCbl)-dependent glutamate mutase toward thiol-directed reagents has been investigated. Iodoacetate specifically alkylates one cysteine residue, Cys-15, in MutS with concomitant irreversible loss of enzyme activity. Cys-15 lies between the conserved residues Asp-14 and His-16, that are believed to coordinate cobalt to form a Co-His-Asp hydrogen-bonded “triaxial” when AdoCbl is bound by the enzyme. Although inactive, carboxymethylated MutS still bound AdoCbl with only a 5-fold increase in apparent $K_a$. To determine whether Cys-15 plays an essential role in catalysis, it was mutated to serine and to alanine. These mutants were active, but both exhibited decreased $V_{max}$ and increased apparent $K_m$ and $K_a$ for AdoCbl. To mimic the effect of carboxymethylation, Cys15 was mutated to aspartate and, as an isosteric control, to asparagine. Neither of these mutants was active: MutS-C15N bound AdoCbl approximately 10-fold weaker than wild type, whereas MutS-C15N bound AdoCbl over 100 times less strongly than wild type. The results demonstrate both coenzyme-binding and catalysis to be very sensitive to mutations at position 15 that could potentially perturb the Co-His-Asp hydrogen-bonding network.

Adenosylcobalamin (coenzyme B$_{12}$, AdoCbl)$^1$-dependent glutamate mutase catalyzes the isomerization of L-glutamate to L-threo-3-methylaspartate as the first step in the fermentation of glutamate by Clostridium tetanomorphum (1). It is one of a group of AdoCbl-dependent enzymes that catalyze unusual carbon-skeleton rearrangements in which a hydrogen on one carbon atom is interchanged with an electron-withdrawing group, X, on an adjacent carbon (Scheme 1) (2, 3).

\[
\begin{array}{c}
H \\
\mid \\
C_1 - C_2 \\
\mid \\
X \\
\mid \\
C_1 - C_2 \\
\mid \\
H
\end{array}
\]

Scheme 1

AdoCbl functions both as the source of free radicals that are required in the mechanism and as the intermediate hydrogen carrier in the rearrangement (4-6). These enzymes are themselves part of an emerging class of enzymes that employ free radicals to effect the cleavage of otherwise unreactive carbon-carbon, carbon-nitrogen, and carbon-oxygen bonds (7, 8).

The first step in all AdoCbl-mediated reactions is homolysis of the coenzyme’s labile cobalt-carbon bond, which serves to “unmask” the catalytic 5′-deoxyadenosyl radical (9). In free solution, the cobalt-carbon bond dissociation energy is between 25 and 30 kcal/mol (10, 11), but to achieve the rates of catalysis seen in the AdoCbl-dependent isomerases, this bond must be substantially weakened when the enzyme binds the coenzyme (6). Studies on free cobalamins, cobinamides, and model alkylcobalt compounds have indicated that both steric compression between the corrin ring and the alkyl group, and the nucleophilicity of the axial nitrogenous ligand to cobalt influence the cobalt-carbon bond strength (12-15). However, the relative importance of these effects in the enzyme-mediated homolytic cleavage of AdoCbl has remained unclear.

Glutamate mutase comprises two readily separable subunits or components designated MutE and MutS (16-18). MutS, a monomer of $M_r$ 14,748, forms part of the cobalamin-binding site and shows sequence similarity to several other cobalamin-dependent enzymes (16, 19), in particular, a key motif, "DXHXXG," is conserved in all these enzymes. The crystal structures of the homologous domains from AdoCbl-dependent methylmalonyl-CoA mutase and MeCbl-dependent methionine synthase have recently been solved (20, 21). A surprising and important feature is that the cofactor is bound in an extended conformation in which the nucleotide tail is displaced and the histidine residue of the DXHXXG motif coordinates the central cobalt atom of the coenzyme from below (Fig. 1). The carboxylate of the aspartate residue forms a hydrogen bond to the histidine to complete a Co-His-Asp “triaxial” analogous to the Fe-His-Asp triad of cytochrome c peroxidase (22). EPR studies of glutamate mutase (23) suggest that cobalt is similarly coordinated by the histidine residue of MutS.

The sensitivity of MutS to thiol-directed reagents was first noted by Switzer and Barker (24). Our investigation of this phenomenon was prompted by the discovery that a protein thiol plays a crucial role as the site of an intermediate, protein-based radical in the mechanism of both the AdoCbl-dependent and iron-dependent ribonucleotide reductases (25, 26). However, recent experiments with isotopically labeled substrates and coenzyme appear to rule out an intermediate protein radical for glutamate mutase (5).

EXPERIMENTAL PROCEDURES

Materials—The construction of the plasmids pmutS and pmutSX, and the purification of MutE and MutS proteins from recombinant Escherichia coli strains have been described previously (18). 3-Methylaspartate was purified from Clostridium tetanomorphum as described...
by Hsiang and Bright (27). AdoCbl was supplied by Fluka Chemical Co.; restriction enzymes and DNA modifying enzymes were purchased from Boeringer Mannheim. Iodo[2-14C]acetic acid was purchased from Amersham Corp. The sources of other materials have either been described previously (18) or were purchased from commercial suppliers.

**Construction of MutS Mutant Proteins**—The mutS gene was excised from the pUC119-based plasmid, pmutS (18) as an EcoRI-PstI fragment and subcloned into the commercial vector pALTER-1 (Promega) to give pALmutS, which was maintained in E. coli JM109. A single-stranded DNA template for mutagenesis was obtained by transfection with helper phage R408. Standard techniques (28) were used to prepare plasmid and single-stranded DNA, and to subclone DNA fragments. Site-directed mutagenesis was performed using the Altered Sites in vitro mutagenesis system (Promega) according to the manufacturer’s protocol. Mutations were introduced into the mutS gene using the following oligonucleotides as primers for second-strand synthesis: MutS-C15A, ATT GGT TCA GAC AAT CAT; MutS-C15G, ATT GGT TCA GAC AAT GAT; MutS-C15S, ATT GGT TCA GAC AAT CAT GCA GTT GG. Mutations were confirmed by DNA sequencing and the mutant mutS genes were then excised from pALmutS as NdeI-SallI fragments and subcloned into pT7-T7 (29) to facilitate over expression of the mutant proteins in E. coli BL12 (DE3) as described previously (18).

**Enzyme Assay**—Glutamate mutase activity was assayed spectrophotometrically, as described previously (1, 18). The concentrations of MutE and MutS were determined by using the following values for their absorption coefficients at 280 nm: MutE ε280 = 56,300 M⁻¹ cm⁻¹, MutS ε280 = 9,380 M⁻¹ cm⁻¹ (18). MutS mutant proteins were assumed to have the same extinction coefficients as wild type protein.

**Carboxymethylation of MutS**—Carboxymethylation was carried out at room temperature in the dark. The reaction contained 0.1 M Tris-Cl, pH 8.5, 0.14 mM MutS (1 mg), 0.65 mM dithiothreitol, and 2.4 mM iodo[2-14C]acetic acid (7160 cpm/nmol) in a total volume of 0.5 ml. At intervals of 0, 2.5, 5, 10, 20, 45, and 100 min, 50 µl of MutS were withdrawn from the reaction and quenched by addition of 50 µl of 0.1 M dithiothreitol in 0.1 M Tris-Cl, pH 8.5. After 15 min in quench solution, 1 µg of MutS was assayed for activity. The remaining quenched reaction was dialyzed overnight against 2 x 2.5 liters 15 mM potassium phosphate buffer, pH 8.0, at 4°C; samples were cleared by centrifugation at 12,000 g for 10 min, and their protein content was determined from absorbance at 280 nm. Extent of radiolabeling was then measured by liquid scintillation counting.

**Measurement of AdoCbl Binding**—Equilibrium gel filtration (30) was used to measure the binding of AdoCbl to glutamate mutase exactly as described previously (18).

**Protein Sequencing**—The N-terminal sequences of HPLC-purified peptides were determined by standard automated sequencing methods on an Applied Biosystems 477 protein sequencer.

**Electrospray Mass Spectrometry**—Proteins were desalted by absorption onto a reverse phase C8 HPLC column and elution with an ascending gradient of acetonitrile, 0.1% trifluoroacetic acid. The sample, 10 pmol/µl, was then analyzed on a Kratos Profile electrospray mass spectrometer.

**RESULTS**

Two properties of the glutamate mutase MutS subunit indicate that cysteine residues may be important in the enzyme mechanism (24). First, MutS is irreversibly inactivated by thiol-directed alkylating agents such as iodoacetate, and secondly, MutS requires prior reduction with either 2-mercaptoethanol or dithiothreitol for activity: the oxidized protein appears to form both inter- and intra-molecular disulfide linkages that render it inactive. To clarify the role of thiols in the mechanism, and to determine whether these two properties are related, we decided to identify the reactive cysteine residue(s) in MutS and examine the effect of mutating this residue on the catalysis.

The inactivation of MutS with iodo[2-14C]acetate at room temperature exhibited the expected time-dependent behavior (Fig. 2). The loss of enzyme activity was well described by pseudo-first order kinetics; the first order rate constant for inactivation, calculated over the first 45 min of the reaction, was 0.15 ± 0.01 min⁻¹. The incorporation of radiolabel into the protein mirrored the loss of activity, although it appeared to proceed slightly faster, k = 0.21 ± 0.02 min⁻¹, this difference is probably not significant. Holo-glutamate mutase exists in equilibrium with dissociated MutS and MutE subunits and coenzyme (18). It was, therefore, not possible to demonstrate protection of MutS by MutE and/or AdoCbl because there is always a pool of free MutS protein available to react with iodoacetate. The maximal extent of labeling was 7600 ± 260 cpm/nmol of MutS which corresponded to 1.06 ± 0.04 labels per molecule of MutS. These results indicate that alkylation of only one cysteine residue is responsible for the inactivation of MutS.

Radiolabeled MutS was subjected to proteolysis using endopeptidase Glu-C from Staphylococcus aureus V8 that cleaves after glutamate residues. This protease was chosen as it should cleave between each of the four cysteine residues in MutS. After digestion, peptides were separated by reverse-phase HPLC on a C8 column using a gradient of acetonitrile in the presence of 0.1% trifluoroacetic acid. The total recovery of radioactivity from the column was ~60%. Radioactivity was associated with several peptides that eluted late in the gradient. The two most highly labeled peptides contained between 50 and 70% of the recovered radioactivity, and were subjected to automated N-terminal sequencing. In both cases sequences corresponded to a peptide starting at Lys-3 (cleaveage after Glu-2) in which Cys-15 had been modified, implying that this is the major site of modification. It was apparent from the digest that more peptides were produced than there were cleavage sites into four regions of the protein corresponding to the four cysteine residues.

**Fig. 1.** Sterevo view of the Co-His-Asp triad for cobalamin bound in methylmalonyl-CoA mutase (20) showing the loop containing residues His-610, Asp-608, and Gly-609. His-610 replaces the benzimidazole moiety that is usually coordinated to cobalt in free solution, is now bound in an extended conformation in a cleft of the protein (not shown in this view). In glutamate mutase the corresponding residues are MutS His-16 and Asp-14, and Cys-15 replaces glycine. The adenosyl portion of the coenzyme was not resolved in the x-ray structure.
sites in the protein; is most likely, therefore, that the various labeled peptides were the result of partial cleavage of the protein, rather than nonspecific labeling. Significantly, Cys-15 lies between the highly conserved residues, Asp-14 and His-16 that form part of the DXHXXG cobalamin-binding motif (16).

The position of Cys-15 between two residues that are important for binding cobalamin suggested that alkylation of this cysteine may have inactivated the enzyme simply because it prevented the coenzyme binding. To test this, the $K_d$ for AdoCbl was measured for the carboxymethylated enzyme using equilibrium gel filtration (30). The apparent $K_d$ for AdoCbl is dependent upon the relative concentrations of MutE and MutS present in the binding assay (18). The apparent $K_d$ for AdoCbl binding to the MutE-carboxymethylated-MutS complex, determined with 12 $\mu M$ MutE and 60 $\mu M$ carboxymethylated-MutS, was 9.5 ± 0.7 $\mu M$, whereas the apparent $K_d$ for the unmodified enzyme, determined under the same protein concentrations, was 1.8 ± 0.2 $\mu M$. Carboxymethylation of MutS, therefore, only raises the apparent $K_d$ for AdoCbl by 5-fold, which does not seem sufficient to explain why the enzyme is inactivated by this modification.

To examine whether Cys-15 plays an essential role in the mechanism, this residue was mutated to both serine and alanine. To investigate the effect of carboxymethylation, Cys-15 was mutated to aspartate, a change designed to probe the effect of introducing negative charge at this position; Cys-15 was also mutated to asparagine as an isosteric control. The purified proteins were analyzed by electrospray mass spectrometry to confirm that the intended mutations had been correctly expressed: in each case the experimentally determined molecular weight was in excellent agreement with that calculated from the sequence (MutS-C15A: calculated $M_r$ 14,716, found 14,713 ± 4; MutS-C15S: calculated $M_r$ 14,732, found 14,728 ± 4; MutS-C15D: calculated $M_r$ 14,760, found 14,763 ± 3; MutS-C15N: calculated $M_r$ 14,759, found 14,762 ± 4).

The kinetic and coenzyme-binding properties of the mutant proteins are summarized in Tables I and II. Both the MutS-C15A and C15S mutants were active in the glutamate mutase assay, demonstrating that Cys-15 does not play an essential role in the mechanism. This is consistent with the fact that Cys-15 is not conserved in other cobalamin-dependent enzymes that share the homologous cobalamin-binding domain and DXHXXG motif (16, 31). The mutant proteins were no longer sensitive to iodoacetate; after incubation with iodoacetate for 1 h at room temperature, conditions that completely inactivate wild type MutS, they remained fully active. This confirms that Cys-15 is the site of modification. For maximal activity both mutants still had to be reduced with dithiothreitol prior to assay, otherwise the proteins exhibited only about 10% activity. Therefore, the sensitivity of the protein to oxidation appears to be due to the formation of disulfide linkages between cysteine residues other than Cys-15.

The MutS-C15A and MutS-C15S mutants differed little in their affinity for MutE and L-glutamate when compared with the wild type enzyme. Both mutants bound MutE slightly less tightly as judged by $K_d$, the apparent dissociation constant under the conditions of the assay, and binding was weakly cooperative, as had previously been found for wild type enzyme (18). The $K_m$ for L-glutamate was, within experimental error, unchanged. The apparent $K_d$ for AdoCbl was measured by equilibrium gel filtration. In both cases the mutants bound the coenzyme significantly more weakly than did wild type enzyme, by 2–3-fold, although this decrease in affinity for AdoCbl was much less than was seen for the inactive MutS-C15D and MutS-C15N mutants. Consistent with this, the apparent $K_m$ for AdoCbl was significantly increased, by about 4-fold for the MutS-C15A mutant and about 6-fold for the MutS-C15S mutants. Finally, although active, these mutants were impaired in catalysis as $k_{cat}$ for either mutant was only about one third that of wild type.

Neither MutS-C15D nor MutS-C15N mutants displayed any detectable glutamate mutase activity. Therefore, the introduction of negative charge at this position does not per se appear to the reason why carboxymethylation inactivates MutS. However, these mutants exhibited a quite striking difference in their ability to bind AdoCbl. The apparent $K_d$ for AdoCbl of MutS-C15N was 17 ± 3 $\mu M$, whereas that for the MutS-C15D mutant was too high to measure; at the highest concentration of AdoCbl (100 $\mu M$) used the mutant showed no sign of saturation. This result was surprising because the carboxymethylated enzyme binds AdoCbl quite well (apparent $K_d$ 9.5 $\mu M$), although in this case the charge is moved further out by two bonds. Possibly, in the case of the MutS-C15D mutation, the introduction of a second negative charge closer to Asp-14 may cause the loop containing the His-Asp pair to undergo a signific-
Conservative mutations cause small increases in the apparent changes in this region of the protein. Thus, even the most
mismatches, our results indicate that catalysis is very sensitive to controls of the reactivity of the coenzyme.

Whether the Co-His-Asp triad is purely structural, or if it also
binds very similarly. However, not all AdoCbl-dependent enzymes share the importance of the His-Asp pair onine synthase and methylmalonyl-CoA mutase cobalamin
cobalt-carbon bond. In particular, the structures of the methi-

fications that introduce good hydrogen-bonding side-chains at
position 15 since all the mutants are impaired to a greater or
serine residues in that enzyme serve to weaken the coordi-
ination of Cys-15 to the cobalt-coordinating histidine, we propose that mutations at this position may perturb the Co-His-Asp hydrogen-bonding network. This might occur if the mutants are able to form alternative hydrogen bonds to histidine or aspartate, otherwise if they form hydrogen bonds to other residues or backbone amid the local con-
formation of the protein may be distorted and cause a less favorable alignment of the Co-His-Asp triad. Certainly, the histidine ligand is important for binding AdoCbl because MutS mutants lacking His-16 bind AdoCbl with significantly reduced affinity.2 Also, recent studies on the analogous Co-His-Asp-Ser hydrogen-bonded quartet of methylcobalamin-dependent methionine synthase have shown that mutation of the aspartate or serine residues in that enzyme serve to weaken the coordi-
nation of cobalt by histidine (33).

Although not essential, cysteine appears to be preferred at position 15 since all the mutants are impaired to a greater or lesser extent in catalysis as well as coenzyme binding. This observation supports the idea that the Co-Asp-His triad is not just a structural motif, but is also plays an important role in catalysis. The results are consistent with a protein-induced trans effect, mediated through the histidine, operating to promote homolysis of the coenzyme’s cobalt-carbon bond. Further experiments are now in progress to elucidate in more detail the mechanism by which glutamate mutase effects homolysis of AdoCbl and to establish more precisely the role of the Co-His-Asp triad in promoting this unusual bond cleavage.

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