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Interaction of Flavodoxin with Cobalamin-Dependent Methionine Synthase†

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ABSTRACT: Cobalamin-dependent methionine synthase catalyzes the transfer of a methyl group from methyltetrahydrofolate to homocysteine, forming tetrahydrofolate and methionine. The *Escherichia coli* enzyme, like its mammalian homologue, is occasionally inactivated by oxidation of the cofactor to cob(II)alamin. To return to the catalytic cycle, the cob(II)alamin forms of both the bacterial and mammalian enzymes must be reductively remethylated. Reduced flavodoxin donates an electron for this reaction in *E. coli*, and *S*-adenosylmethionine serves as the methyl donor. In humans, the electron is thought to be provided by methionine synthase reductase, a protein containing a domain with a significant degree of homology to flavodoxin. Because of this homology, studies of the interactions between *E. coli* flavodoxin and methionine synthase provide a model for the mammalian system. To characterize the binding interface between *E. coli* flavodoxin and methionine synthase, we have employed site-directed mutagenesis and chemical cross-linking using carbodiimide and *N*-hydroxysuccinimide. Glutamate 61 of flavodoxin is identified as a cross-linked residue, and lysine 959 of the C-terminal activation domain of methionine synthase is assigned as its partner. The mutation of lysine 959 to threonine results in a diminished level of cross-linking, but has only a small effect on the affinity of methionine synthase for flavodoxin. Identification of these cross-linked residues provides evidence in support of a docking model that will be useful in predicting the effects of mutations observed in mammalian homologues of *E. coli* flavodoxin and methionine synthase.

Flavodoxins are electron-transfer proteins that contain an FMN[1] prosthetic group (*1*). They serve as electron donors in both anaerobic and aerobic bacteria (*1*, *2*). In *Escherichia coli*, the known electron acceptors include anaerobic ribonucleotide reductase (*3*), biotin synthase (*4*), pyruvate formate lyase (*5*), and cobalamin-dependent methionine synthase (*6*).

Cobalamin-dependent methionine synthase catalyzes the transfer of a methyl group from methyltetrahydrofolate to homocysteine to form tetrahydrofolate and methionine. In the primary turnover cycle (Figure 1), the cob(I)alamin cofactor of methionine synthase is methylated by methyltetrahydrofolate to form the methylcobalamin cofactor. This methyl group is transferred to homocysteine to form methionine, thus returning the cofactor to the cob(I)alamin form. Every 100−2000 cycles of this reaction, however, the cob(I)alamin cofactor is oxidized to the cob(II)alamin state (*7*, *8*). The cob(II)alamin form is inactive in primary turnover. To return to the catalytic cycle, the enzyme must be reductively remethylated. In the reactivation cycle, AdoMet replaces methyltetrahydrofolate as the methyl donor (*9*, *10*), and reduced flavodoxin serves as the electron donor (*6*, *8*). Several lines of evidence suggest that the cobalamin can only interact with one substrate-binding module at a time (*11*). Thus, we believe that the activation conformation and the turnover conformations of methionine synthase are mutually exclusive.

Methionine synthase is a modular protein: the first 352 residues comprise a homocysteine-binding region (*12*), residues 353−649 are involved in the binding of methyltetrahydrofolate (*12*), residues 650−896 bind the cobalamin cofactor (*13*), and the carboxy-terminal 38 kDa region (residues 897−1227) is required for reactivation and binds AdoMet (*7*). We have been able to generate a series of fragments that retain partial function. A 71 kDa fragment (residues 2−649) contains the homocysteine and methyltetrahydrofolate binding modules and catalyzes methyl transfer to and from exogenous cobalamin (*12*). A 98 kDa fragment (residues 2−896) comprises both the substrate-binding regions and the cobalamin-binding module and is capable of turnover using the endogenous cobalamin cofactor (*7*). The C-terminal 38 kDa fragment binds AdoMet (*7*), and in

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[1] Abbreviations: FMN, flavin mononucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; AdoMet, *S*-adenosyl-L-methionine; EDTA, ethylenediaminetetraacetic acid; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; NHS, *N*-hydroxysuccinimide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; PVDF, polyvinylidene fluoride Immobilon-P; Hcy, homocysteine; Met, methionine; H_4folate, tetrahydrofolate; CH_3-H_4folate, methyltetrahydrofolate; AdoHcy, *S*-adenosylhomocysteine; Fld, flavodoxin; MetH, cobalamin-dependent methionine synthase.

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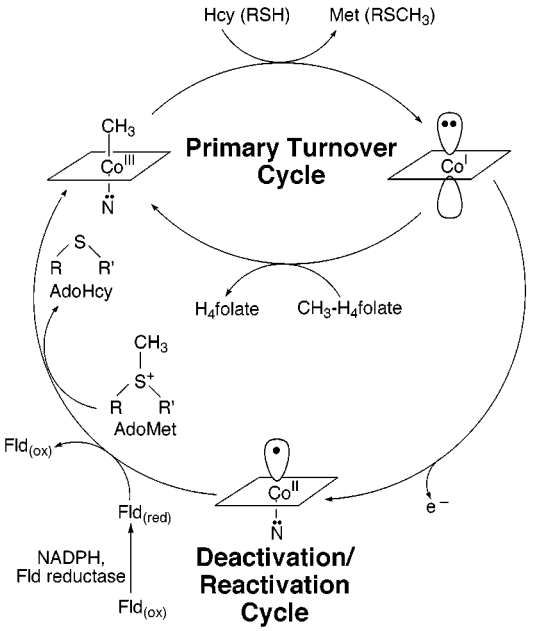

FIGURE 1: Reactions catalyzed by cobalamin-dependent methionine synthase. In primary turnover, homocysteine is methylated by methylcobalamin, yielding methionine and cob(I)alamin. Methylcobalamin is regenerated by reaction with methyltetrahydrofolate, yielding tetrahydrofolate. Cob(I)alamin may be inactivated by oxidation to cob(II)alamin. Return to the catalytic cycle requires reductive methylation; flavodoxin donates the electron, and AdoMet provides the methyl group. Under aerobic growth conditions, flavodoxin semiquinone is thought to be the predominant reduced species.

this paper, we demonstrate that it also contains important determinants for flavodoxin binding.

The interactions of flavodoxin with its physiological partner ferredoxin/flavodoxin-NADP$^+$ oxidoreductase have been examined in detail (*14*); during aerobic growth, this enzyme is responsible for reduction of flavodoxin (*6*). There are still no published structures of complexes between flavodoxin and its physiological partners. However, some determinants of molecular recognition between flavodoxin and its protein partners have been elucidated. Because flavodoxins are very acidic proteins with pI values of 3.1−5.1, electrostatic interactions between flavodoxin and basic regions on its partners are thought to be important in binding (*15*, *16*). Interactions of flavodoxin with ferredoxin/flavodoxin-NADP$^+$ oxidoreductase and methionine synthase are dependent upon ionic strength, suggesting a role for electrostatic interactions (*17*, *18*). Structural studies of ferredoxin/flavodoxin-NADP$^+$ oxidoreductase reveal a depression near the binding site for flavin adenine dinucleotide that could provide both charge and shape complementarity to accommodate the face of flavodoxin where the FMN cofactor is bound (*19*, *20*).

Methionine synthase reductase, a protein containing domains with a significant degree of homology to flavodoxin and ferredoxin/flavodoxin-NADP$^+$ oxidoreductase, has been proposed to be the functional homologue for reactivation of methionine synthase in humans (*21*), and we anticipate that the features controlling molecular recognition of methionine synthase by flavodoxin will be conserved in higher organisms. Mutations in the reductase appear to cripple the activity of methionine synthase, since patients with these mutations exhibit elevated plasma homocysteine and homocysteinuria (*21*, *22*). Definition of the interactions between flavodoxin and *E. coli* methionine synthase might be expected to rationalize the phenotypes associated with mutations of human methionine synthase reductase and to delineate the region where it interacts with methionine synthase.

The residues critical to the interaction between flavodoxin and *E. coli* methionine synthase have not been defined. Because the reduced flavin of flavodoxin donates an electron to the cob(II)alamin form of methionine synthase, which then accepts a methyl group from AdoMet, the flavodoxin−methionine synthase interface is likely to involve contacts between flavodoxin and both the cobalamin- and AdoMet-binding modules of methionine synthase.

To characterize the binding interface between flavodoxin and methionine synthase, we have utilized chemical cross-linking. A covalent complex in which flavodoxin is cross-linked to the 38 kDa activation domain of methionine synthase has been generated. Glutamate 61 of flavodoxin has been identified as a cross-linking residue, and lysine 959 of methionine synthase is assigned as its cross-linking partner. From studies of mutants, lysine 1035 of methionine synthase is also implicated in binding flavodoxin.

MATERIALS AND METHODS

Materials. All plasmids were prepared using the Promega Wizard Plus Minipreps DNA purification kit (Madison, WI). Restriction enzymes, *Pfu* polymerase, and T4 DNA ligase were supplied by Promega and New England Biolabs (Beverly, MA). Sequencing grade endoproteinase LysC was purchased from Promega. All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of Proteins. Purified recombinant wild-type *E. coli* methionine synthase was prepared as previously described from *E. coli* K-12 strain XL1-Blue/pKF5a using anion exchange chromatography (*23*). The 98 and 38 kDa regions of this protein were obtained by limited tryptic proteolysis of methionine synthase and purified by anion exchange and size exclusion chromatography (*24*). The 71 kDa methionine synthase fragment was obtained from *E. coli* K-12 strain XL1-Blue/pCWG-02 and purified by anion exchange chromatography as described by Goulding and coworkers (*12*). Wild-type *E. coli* flavodoxin was prepared from *E. coli* K-12 strain XL1-Blue/pDH01 and purified by anion exchange chromatography (*3*).

Generation of Histidine-Tagged E. coli Flavodoxin. Carboxy-terminal His$_6$-tagged *E. coli* flavodoxin (His$_6$flavodoxin) was generated using PCR-based primer overlap mutagenesis techniques (*25*). In conjunction with the template flavodoxin plasmid pDH01, the complementary mutant primers CTCAATGCC**CTCGAG**TCTAGAATTC and GAATTCTAGA**CTCGAG**GGCATTGAG were used to create an insert in which a *Xho*I restriction site (bold text) replaces the stop codons at the carboxy terminus of the flavodoxin gene. These primers are located at nucleotides 899−913 of the nucleotide GenBank sequence M59426 for *E. coli fldA* flavodoxin. The external primers were located at nucleotides 711−727 in *fldA* and within the vector 105−130 nucleotides downstream of the flavodoxin gene. The insert was introduced into the flavodoxin gene using *Bst*BI and *Pst*I restriction sites located at nucleotide 760 in *fldA* and 52 nucleotides downstream of the flavodoxin gene. This construct was sequenced between

the *Bst*BI and *Pst*I restriction sites using the Sequenase protocol (United States Biochemicals, Cleveland, OH) to ensure that no other mutations were present. The entire flavodoxin gene containing the *Xho*I site was then subcloned from this construct into the pET23-b vector (Novagen, Madison, WI) using the restriction enzymes *Nde*I and *Xho*I to create the plasmid pDH7. This plasmid enables expression of flavodoxin with the addition of the carboxy-terminal sequence Leu-Glu-His_6.

The His_6flavodoxin was expressed in cells of *E. coli* K-12 strain XL1-Blue (Stratagene, La Jolla, CA) containing the pDH7 plasmid. Bacteria were grown at 37 °C and harvested as previously described (*3*). Cells were resuspended to a density of 0.25−0.5 g of cells/mL in 40 mM imidazole and 20 mM sodium phosphate (pH 7.4), with N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (10 μg/mL) and phenylmethanesulfonyl fluoride (50 μg/mL). Cells were then lysed by sonication or by using a French press. Unbroken cells and membranes were removed by ultracentrifugation for 60 min at 100000*g*.

Nickel affinity chromatography was used to purify the His_6flavodoxin. Sodium chloride (500 mM) was added to the lysate, which was then applied to a HiTrap Chelating nickel−agarose column (Amersham Pharmacia, Piscataway, NJ) that was equilibrated with 40 mM imidazole, 500 mM sodium chloride, and 20 mM sodium phosphate (pH 7.4). The column was washed with 4 column volumes of this buffer. The His_6flavodoxin eluted with 150 mM imidazole, 500 mM sodium chloride, and 20 mM sodium phosphate (pH 7.4). The purified His_6flavodoxin was exchanged into 10 mM potassium phosphate (pH 7.4) containing 0.1 mM EDTA and 1 mM dithiothreitol. The protein concentration was calculated using the absorbance of bound FMN (ϵ_{466} = 8250 M^{-1} cm^{-1}) (*5*). The yield ranged from 60 to 100 mg of purified His_6flavodoxin per liter of cell culture.

Mutagenesis of Candidate Cross-Linking Residues of Methionine Synthase. To create methionine synthase mutants, site-directed mutagenesis was performed using the QuikChange protocol (Stratagene). The methionine synthase plasmid pKFsyn41 was employed as the template (*26*). This plasmid specifies the wild-type methionine synthase protein but uses a synthetic gene for the 38 kDa region to facilitate mutagenesis. The complementary primers GGTCGCTGGCCGG-**TACC**TATCCGCGCATTCTAG and CTAGAATGCGCG-GATA**GG**T**A**CCGGCCAGCGACC were used to generate the Lys959Thr mutation; primers CGTCAACAGACCGAAA-**CT**ACAGGCTTCGCTAAC and GTTAGCGAAGCCTG-T**AG**TTTCGGTCTGTTGACG were employed in generation of the Lys1035Thr mutation, and primers CCACCCGGAT-TC**CA**C**G**TACTACGCTGTAGC and GCTACAGCGTAG-TAC**G**TCGAATCCGGGTGG were used to generate the Lys1888Thr mutation. These primers, with mutated nucleotides shown in bold text, are located at nucleotides 3083−3115, 3310−3342, and 3771−3800 of GenBank nucleotide sequence J04975 for *E. coli metH*. The mutant plasmids were sequenced (University of Michigan DNA Sequencing Core Facility, Ann Arbor, MI) in the region surrounding the incorporated mutation to ensure that no other base changes had occurred. Mutant plasmids were then cut with restriction enzymes within the sequenced region (*Xba*I and *Bsi*WI for Lys959Thr, *Bsi*WI and *Bst*EII for Lys1035Thr, and *Xma*I and *Nde*I for Lys1188Thr). The mutant inserts were ligated back into the identically-cut pKFsyn41 vector to create the final mutant plasmids pK959T, pJSK1035T, and pJSK1188T. This step eliminated the need for sequencing entire mutant plasmids since both the cut pKFsyn41 vector and the inserts have confirmed sequences. For creation of the Lys959Thr/Lys1035Thr double mutant, the pK959T and pJSK1035T plasmids were cut with *Xba*I and *Bsi*WI enzymes. The mutation-containing fragments were then ligated to create the pK959T/K1035T plasmid. All mutant plasmids are maintained as transformants in *E. coli* K-12 strain XL1-Blue.

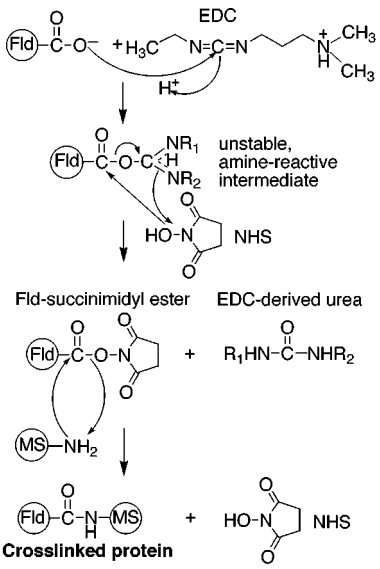

FIGURE 2: Summary of the cross-linking chemistry. Acidic residues on one protein (flavodoxin, Fld) are activated for cross-linking by reaction with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC). A more stable protein−succinimidyl ester intermediate forms by reaction with *N*-hydroxysuccinimide (NHS). In a second step, lysine residues on the partner protein (methionine synthase, MS) attack the succinimidyl ester, yielding a cross-linked protein.

The methionine synthase mutant proteins were expressed and purified in the same manner as the wild-type methionine synthase (*23*). Methionine synthase activity was measured at 37 °C using a spectrophotometric methyltetrahydrofolate−homocysteine methyltransferase assay (*27*). This fixed time point assay measures the extent of conversion of methyltetrahydrofolate to tetrahydrofolate by derivatization of tetrahydrofolate to methenyltetrahydrofolate, which absorbs at 350 nm. The 38 kDa regions of the mutant proteins were generated by tryptic proteolysis and purified in the same manner as the wild-type 38 kDa fragment (*24*).

Cross-Linking Experiments. To cross-link flavodoxin and methionine synthase, the reagents 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were employed in a two-step reaction at room temperature (Figure 2). First, 40 μM flavodoxin (wild-type or His_6-tagged) was incubated for 5 min with 5 mM EDC and 5 mM NHS in 20 mM 2-*N*-(morpholino)ethanesulfonic acid buffer (pH 6.0). This reaction was quenched by addition of 20 mM β-mercaptoethanol, and cob(II)alamin methionine synthase or a fragment of this protein (8 μM) was then added. After 2.5 h, the reaction was terminated by addition of 10 mM hydroxylamine, which regenerates the acidic residues that had been activated but did not cross-link. Samples were exchanged into 0.3 mM EDTA in 10 mM potassium phosphate (pH 7.2) using Microcon 30 membrane centrifugation units (Millipore, Bedford, MA) and then were

analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) using 10 or 15% polyacrylamide gels with Coomassie blue staining. For densitometric analyses, gels were scanned using a Hewlett-Packard ScanJet 4c scanner and analyzed using NIH Image 1.60 software.

Purification of Cross-Linked Proteins. The cross-linked complex between methionine synthase and His_6flavodoxin was isolated using size exclusion chromatography to eliminate unreacted His_6flavodoxin and nickel affinity chromatography to selectively retain the cross-linked complex and exclude unreacted methionine synthase. In this procedure, excess flavodoxin was cross-linked with methionine synthase in the cob(II)alamin form (235 mg, 1.73 μmol). The sample was concentrated to 16 mL, and thirteen 1.25 mL aliquots were separated on a Superose 12 HR 10/30 Pharmacia FPLC column equilibrated with 200 mM sodium chloride in 20 mM sodium phosphate (pH 7.4). A flow rate of 0.5 mL/min was employed. Fractions containing the cross-linked protein were combined and added in two aliquots to a 5 mL HiTrap Chelating nickel−agarose column. This column was washed with 4 column volumes of 200 mM sodium chloride in 20 mM sodium phosphate (pH 7.4). The cross-linked protein was then eluted in the same buffer with 175 mM imidazole. The cross-linked protein was concentrated and exchanged (by size exclusion chromatography) into 10 mM potassium phosphate (pH 7.2) containing 0.3 mM EDTA. The yield was 20.4% (55.2 mg, 0.35 μmol of purified cross-linked protein). The cross-linked species was purified to greater than 95% homogeneity as determined by Coomassie blue staining.

The cross-linked complex between the 38 kDa fragment of methionine synthase and His_6flavodoxin was purified from the unreacted parent proteins by size exclusion chromatography. In this procedure, His_6flavodoxin was cross-linked with the 38 kDa protein (22 mg, 0.579 μmol). The sample was concentrated to 1.5 mL, and two 0.75 mL aliquots were separated on a Superdex 75 prep grade 35/600 Pharmacia FPLC column equilibrated with 0.3 mM EDTA, 500 mM potassium chloride, and 50 mM potassium phosphate (pH 7.2). A flow rate of 0.9 mL/min was used. The yield was 23.5% (8 mg, 0.136 μmol of purified cross-linked protein).

Characterization of Cross-Linked Species. Masses and N-terminal sequences were obtained for the purified cross-linked complexes between His_6flavodoxin and either methionine synthase or the 38 kDa activation domain of methionine synthase. Mass analysis was performed using a PerSpective Voyager Elite matrix-assisted laser desorption ionization mass spectrometer. N-Terminal amino acid sequencing was performed by the University of Michigan Protein and Carbohydrate Structure Facility (Ann Arbor, MI) using a Perkin-Elmer Applied Biosystems automated Edman degradation sequenator (model 494HT) with phenylthiohydantoin amino acid analysis.

Intact flavodoxin is resistant to digestion. Therefore, prior to enzymatic digestion, the FMN cofactor was removed from the cross-linked protein using trichloroacetic acid precipitation as outlined by Edmondson and Tollin (*28*). The cross-linked complex was then resuspended in 100 mM tris(hydroxymethyl)aminomethane buffer (pH 8.5) and digested with 1.2% (w/w) LysC as follows: the cross-linked complex (100 μg/100 μL) was diluted 1:1 with 9 M urea in 100 mM tris(hydroxymethyl)aminomethane buffer (pH 8.0) and incubated with LysC (1.2 μg) for 17 h at 37 °C. Control samples of His_6flavodoxin and the 38 kDa protein were treated in the same manner. The resulting peptides were separated by SDS−PAGE and transferred to a PVDF membrane (Millipore) by electrophoresis at 50 V for 1 h in 10 mM CAPS buffer (pH 10.5) containing 3.25 mM dithiothreitol and 15% methanol. The blot was stained with Coomassie blue. The appropriate bands were excised and subjected to N-terminal sequence analysis. Mass analyses of the digests were performed prior to blotting.

Isothermal Titration Calorimetry. Isothermal titration calorimetry experiments were performed using a Calorimetry Sciences Corp. (Pleasant Grove, UT) model 4200 isothermal titration calorimeter. Protein solutions were exchanged into 50 mM potassium phosphate buffer (pH 7.0) with 0.1 mM EDTA and degassed prior to experiments. Experiments were conducted at 25 °C. In a typical titration, 10 μL aliquots of 1.2−2 mM flavodoxin were titrated into 1.28 mL of 150 μM hydroxocobalamin methionine synthase at 360 s intervals. For the Lys1035Thr mutant, concentrations of flavodoxin (3 mM) and methionine synthase (300 μM) were increased because of weak binding. Control experiments showed negligible heats of dilution for injection of flavodoxin into buffer. For each injection of flavodoxin, the reaction heat was observed as a power signal (microjoules per second). Negative power corresponds to an endothermic reaction in which power input is required to maintain sample and reference compartments at identical temperatures. Integration of the power peaks over the time of an injection yields heat (microjoules) per injection. These data were fit to eq 1 (*29*) using Bindworks 3.0.78 software (Calorimetry Sciences Corp.).

$$Q = V\Delta H\{[1 + MnK + KL - \sqrt{(1 + MnK + LK)^2 - 4MnK^2L}]/2K\} \quad (1)$$

This equation assumes n independent flavodoxin-binding sites per molecule of methionine synthase, where Q is the reaction heat, V is the reaction volume, ΔH is the enthalpy of binding, M is the total macromolecule concentration, K is the association constant, and L is the total ligand concentration. From the relationships shown in eqs 2 and 3, the free energy of binding ΔG and the entropy of binding ΔS can be determined.

$$\Delta G = -RT \ln K \quad (2)$$

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

RESULTS

The Complex between Flavodoxin and Methionine Synthase Can Be Cross-Linked. To cross-link flavodoxin with methionine synthase, the reagents 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were used. Carbodiimide is an attractive reagent because it reacts with acidic functional groups, and flavodoxin is a very acidic protein. This reagent has been successfully employed in cross-linking flavodoxin with ferredoxin/flavodoxin-$NADP^+$ oxidoreductase (*30*−*34*). NHS was included to form a more stable succinimidyl ester intermediate and thus increase the cross-linking yield (*35*).

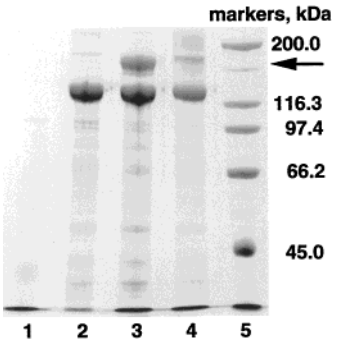

FIGURE 3: Cross-linking flavodoxin and methionine synthase. Acidic residues on one protein were activated with 5 mM EDC and 5 mM NHS for 5 min. Activation was quenched, and the second protein was added and incubated for 2.5 h. Samples were analyzed by SDS−PAGE. Lanes 1 and 2 were loaded with protein from control reaction mixtures containing only His_6flavodoxin and methionine synthase, respectively. In the sample analyzed in lane 3, acidic residues on flavodoxin were activated for cross-linking and then methionine synthase was added. The cross-linked complex between flavodoxin and methionine synthase is shown by the arrow. In the sample analyzed in lane 4, acidic residues on methionine synthase were activated and then flavodoxin was added; no complex of the correct size was observed.

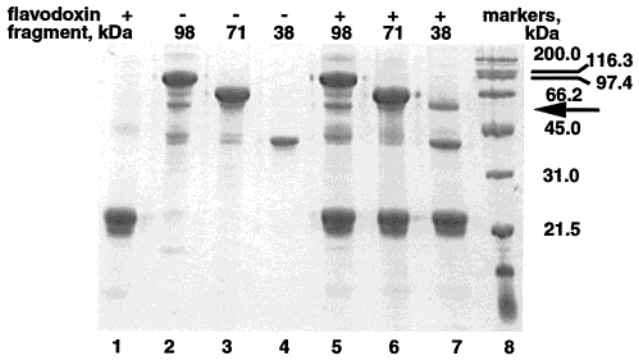

FIGURE 4: Cross-linking flavodoxin and fragments of methionine synthase. His_6flavodoxin was activated with 5 mM EDC and 5 mM NHS for 5 min. The reaction was quenched, and various fragments derived from methionine synthase were added and incubated for 2.5 h. Samples were analyzed by SDS−PAGE. Lanes 1−4 were loaded with protein from control reaction mixtures containing only His_6flavodoxin, or the 98, 71, and 38 kDa fragments of methionine synthase, respectively. Lanes 5−7 contained His_6flavodoxin reacted with the 98, 71, and 38 kDa fragments, respectively. The position of the cross-linked complex containing His_6flavodoxin and the 38 kDa fragment is shown by the arrow.

A two-step procedure was employed to cross-link flavodoxin with methionine synthase in good yields (Figure 2). In this two-step reaction scheme, order-of-addition experiments can identify which protein is the acidic partner and which is the basic partner. In the first reaction step, acidic residues of one protein are activated by reaction with EDC and NHS. Then a partner protein is added. In a complex in which the acidic residue of the activated protein is in proximity to a basic residue of the partner protein, an isopeptide bond forms and a cross-linked species is observed. Reaction in the opposite order usually does not yield a cross-linked product because the activated residues are inappropriately positioned for interaction.

Treatment of the 21 kDa His_6flavodoxin with EDC and NHS followed by addition of the 136 kDa methionine synthase protein results in the formation of a higher-molecular mass protein species, a cross-linked complex of flavodoxin and methionine synthase (Figure 3, lane 3). N-Terminal sequencing of the purified species confirms the presence of both flavodoxin and methionine synthase; mass spectrometry indicates a 1:1 complex by providing a molecular mass of 157 599 Da (calculated mass for 1:1 stoichiometry of 156 855 Da). Excess mass may represent unquenched EDC- or NHS-modified acidic residues. A similar species forms upon reaction of wild-type flavodoxin (20 kDa) with methionine synthase (data not shown), confirming that the C-terminal Leu-Glu-His_6-tag sequence (which is located on the opposite side of the flavodoxin molecule from the flavin) does not perturb cross-linking. Reaction of methionine synthase with EDC and NHS followed by subsequent addition of flavodoxin, however, does not yield a cross-linked species of the correct size (Figure 3, lane 4). Thus, the two-stage cross-linking protocol identifies flavodoxin as the acidic partner protein and methionine synthase as the basic partner in the cross-linking reaction.

Flavodoxin Cross-Links with the 38 kDa Activation Fragment. To characterize the locus of the cross-link as well as the specificity of the interaction, cross-linking reactions were performed with the 98, 71, and 38 kDa fragments of methionine synthase, which comprise amino acids 2−896, 2−649, and 897−1227, respectively (Figure 4). No reaction is observed with His_6flavodoxin and the 98 or 71 kDa fragment. His_6flavodoxin reacts with the 38 kDa fragment, however, resulting in formation of a cross-linked protein. This species was purified, and mass spectrometry again revealed a 1:1 complex with a molecular mass of 58 569 Da (calculated mass for 1:1 stoichiometry of 58 470 Da). N-Terminal sequencing confirmed the presence of both flavodoxin and the 38 kDa fragment of methionine synthase. Cross-linking between flavodoxin and the 38 kDa fragment of methionine synthase is consistent with the idea that there are extensive contacts between these participants in the reactivation reaction of methionine synthase.

Mutagenesis of Candidate Cross-Linking Residues and Effects of Mutations on Cross-Linking. Lysines are generally considered to be the only basic amino acids that are involved in cross-linking by reacting with succinimidyl esters (*36*). The 38 kDa region of methionine synthase has three surface lysines in proximity to the bound AdoMet (Figure 5). These lysines, Lys959, Lys1035, and Lys1188, are candidate cross-linking residues. Mutagenesis was employed to examine the role of these lysines in cross-linking and flavodoxin binding. The methionine synthase mutants Lys959Thr, Lys1035Thr, Lys1188Thr, and Lys959Thr/Lys1035Thr were generated. Threonine lacks the amine group required for the cross-linking reaction and is a preferred in vivo substitution for lysine (*37*). These mutants were expressed and purified. The Lys959Thr, Lys1035Thr, and Lys959Thr/Lys1035Thr proteins retain 95−100% of the wild-type activity level in the spectrophotometric methyltetrahydrofolate−homocysteine methyltransferase assay, which uses a chemical reducing system (dithiothreitol and hydroxocobalamin) to substitute for flavodoxin. The Lys1188Thr methionine synthase retains 86% of wild-type activity. These data suggest that the mutant methionine synthases are properly folded.

Cross-linking of the mutant proteins to flavodoxin was examined using the 38 kDa fragments derived from each mutant (Figure 6). The Lys1188Thr protein resembles wild-

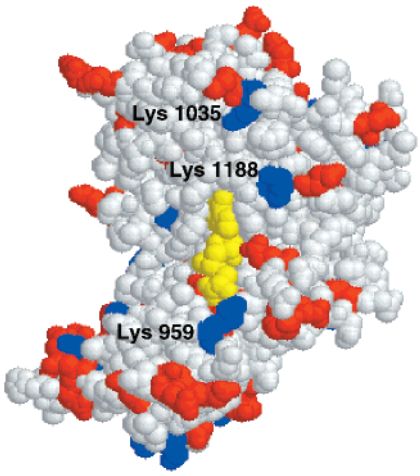

FIGURE 5: Surface view of the 38 kDa AdoMet-binding fragment of methionine synthase. The AdoMet of the 38 kDa AdoMet-binding fragment is shown in yellow. Acidic glutamate and aspartate residues are shown in red, and lysines are displayed in blue.

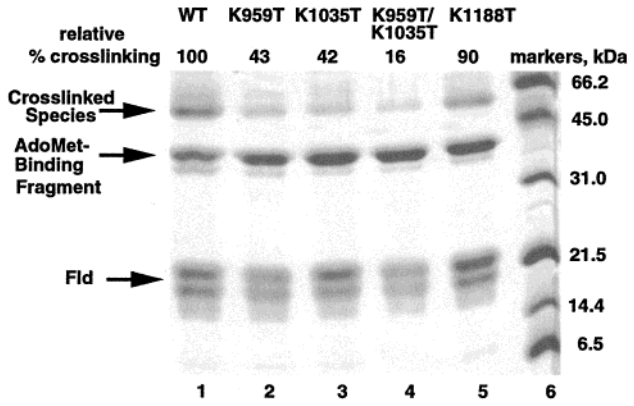

FIGURE 6: Cross-linking flavodoxin with methionine synthase mutants. His_6flavodoxin was activated with 5 mM EDC and 5 mM NHS for 5 min. The reaction was quenched, and the 38 kDa methionine synthase fragment was added and incubated for 2.5 h. Samples were analyzed by SDS−PAGE and bands quantified using NIH Image 1.60 software. Lane 1 was loaded with a protein from a control reaction mixture containing His_6flavodoxin and wild-type 38 kDa protein. Lanes 2−5 contained His_6flavodoxin reacted with Lys959Thr, Lys1035Thr, Lys959Thr/Lys1035Thr, and Lys1188Thr mutant 38 kDa fragments.

type methionine synthase in cross-linking efficiency, while a reduced level of cross-linking was seen with the Lys959Thr (43%) and Lys1035Thr (42%) mutant proteins. In the double mutant Lys959Thr/Lys1035Thr, the level of cross-linking was further decreased to 16% of the wild-type level. The full-length methionine synthase mutants exhibited approximately the same extent of cross-linking to flavodoxin as their 38 kDa fragment counterparts. A decreased level of cross-linking suggests either that the mutated lysine may have formed a cross-link or that the mutation results in a decreased level of binding of flavodoxin to methionine synthase. These results suggest the involvement of Lys959 and Lys1035, but not Lys1188, in binding or cross-linking chemistry.

Mutagenesis of Candidate Cross-Linking Residues and Effects of Mutations on Binding. Isothermal titration calorimetry was performed to determine whether Lys959Thr and Lys1035Thr methionine synthases were deficient in flavodoxin binding. Flavodoxin was titrated into the hydroxocobalamin form of methionine synthase because this enzyme forms tight complexes with flavodoxin (*18*). Titration revealed an endothermic, and thus entropically driven, binding (data not shown), perhaps due to water release associated with changes in the conformation of methionine synthase on flavodoxin binding. Analysis of the number of binding sites was complicated by a decrease in activity of methionine synthase upon conversion from methylcobalamin to hydroxocobalamin forms. This conversion is performed by aerobic reaction with homocysteine, which can result in enzyme damage by exposure to peroxide (*38*). In analyzing our data, we observed a rough correlation between the stoichiometry of flavodoxin binding to methionine synthase (n) and the residual activity of methionine synthase, and so we assumed that the inactive enzyme does not bind flavodoxin. After correction for the activity of methionine synthase, approximately one flavodoxin-binding site per methionine synthase is observed. As seen in Table 1, the dissociation constant of 7.8 μM for binding of flavodoxin to Lys959Thr enzyme was only slightly higher than the K_d of 1.4 μM for interaction with wild-type methionine synthase. Since cross-linking experiments were performed with 40 μM flavodoxin, both forms of methionine synthase would be expected to be fully ligated. These data suggest that the Lys959Thr mutant is deficient in cross-linking chemistry rather than in flavodoxin binding. The dissociation constant for flavodoxin binding to the Lys1035Thr protein (71.8 μM) was markedly elevated compared to that of the wild-type enzyme. Thus, a decreased level of cross-linking of Lys1035Thr methionine synthase probably results from a diminished level of binding of flavodoxin under the conditions used in our experiments.

Identification of Cross-Linked Residues. To determine the cross-linking location and to confirm a role for Lys959 in cross-linking, proteolysis of the cross-linked protein was performed. Fragments were analyzed by N-terminal sequencing and mass spectrometry. The cross-linked region can be identified because a new, larger peptide incorporating fragments from each parent protein will be observed. Endoproteinase LysC cleaves specifically at the carboxy-terminal side of lysine residues. This protease was chosen because digestion patterns are particularly informative when lysine is a cross-linking residue. In the cross-linked peptide, LysC would not be expected to cleave at the cross-linked lysine, which would not be accommodated in the protease active site.

Comparison of the digests of flavodoxin, the 38 kDa fragment of methionine synthase, and the cross-linked complex reveals two striking differences (Figure 7). First, a 7 kDa peptide that is present in the digest of the 38 kDa fragment is absent in the cross-linked sample. This peptide has the N-terminal sequence of KKPRTP (starting at residue 897 of methionine synthase). The 7 kDa mass is consistent with the predicted methionine synthase LysC peptide residues 897−959 (calculated mass of 7281 Da). The absence of a peptide at this position in the cross-linked sample suggests that methionine synthase residues 897−959 are cross-linked to a flavodoxin peptide, possibly at lysine 959. Second, the cross-linked digest contains a 14 kDa band that was not present in the control digests. This new band is a candidate for the cross-linked peptide. Upon sequence analysis, two amino acids (one from flavodoxin and one from methionine synthase) were released in each cycle as would be expected for two cross-linked peptides.

Table 1: Thermodynamic Parameters for Flavodoxin−Hydroxocobalamin Methionine Synthase Binding As Determined by Isothermal Titration Calorimetry

MetH	K_d (μM)	ΔH (kJ/mol)	n	activity (%)	n, corrected	ΔG (kJ/mol)	ΔS (J mol^{-1} K^{-1})
WT	1.4 ± 0.6	39.2 ± 13	0.54 ± 0.03	69	0.78 ± 0.03	−33.7 ± 1.2	244 ± 41
K959T	7.8 ± 0.2	42.2 ± 1.1	0.32 ± 0.01	39	0.82 ± 0.02	−29.2 ± 0.1	239 ± 3
K1035T	71.8 ± 4.7	21.4 ± 0.2	0.19 ± 0.01	43	0.45 ± 0.01	−23.7 ± 0.2	151 ± 1
K1188T	4.0 ± 0.9	34.0 ± 6.3	0.59 ± 0.26	54	1.09 ± 0.26	−30.9 ± 0.6	218 ± 20

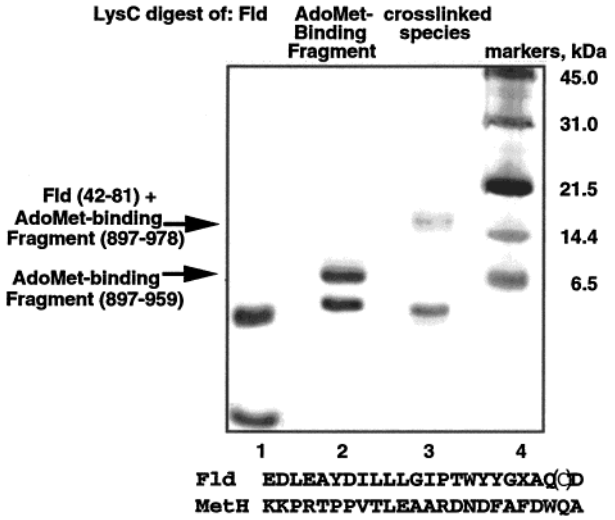

FIGURE 7: Proteolytic digestion of the cross-linked complex of flavodoxin with the 38 kDa activation domain of methionine synthase. Purified His_6flavodoxin (lane 1), the 38 kDa fragment (lane 2), and the complex between the two (lane 3) were digested with endoproteinase LysC. The resulting peptides were separated by SDS−PAGE and transferred to a PVDF membrane. The blot was stained with Coomassie blue. The N-terminal amino acid sequence of the cross-linked peptide from the complex between His_6flavodoxin and the 38 kDa activation domain (upper arrow) is shown below the image of the gel. The N-termini of the cross-linked peptide are flavodoxin residue 42 and methionine synthase residue 897. The **X** denotes the position of Glu61 in sequencing; in that cycle, only phenylalanine 916 from methionine synthase was observed. The absence of Glu61 in this cycle indicates that it is the cross-linked acidic residue. The (C) signifies a cysteine of flavodoxin that could not be observed because the cysteines of the peptide were not derivatized prior to sequencing.

From the N-terminal amino acid sequencing and mass analyses, we were able to determine the identity of the cross-linked residues. When two peptides are cross-linked, sequence analysis should reveal two distinct amino acids during each cycle except at the site of the cross-link. At this point, the residue that participates in the cross-link will be absent, and hence, only the "free" residue will be detected. This is because the cross-linked peptide is composed of two fragments in which the number of residues from the N-terminus to the cross-linking point generally differs. The two N-termini are EDLEAYDILLLGIPTWYYG**X**AQ(C)D, starting at residue 42 of flavodoxin, and KKPRTPPVTLEAARDNDFAFDWQA, starting at residue 897 of methionine synthase. The (C) signifies a cysteine that could not be observed by the sequencing method since cysteines were not derivatized. The **X** denotes a position where only Phe916 from methionine synthase was observed, and no residue was released from flavodoxin. This position in sequence corresponds to Glu61 of flavodoxin, thereby identifying it as the acidic cross-linking residue.

The size of the cross-linked peptide helps to assign the basic partner of Glu61. The cross-linked peptide is 14 kDa, consistent with the calculated mass of 14 253 Da for residues 42−81 of flavodoxin being cross-linked to residues 897−978 of methionine synthase. Several lines of evidence support the identification of Lys959 as the basic partner that participates in the cross-link with Glu61 of flavodoxin. First, it is the only lysine other than the C-terminal residue within this methionine synthase peptide that lies downstream of the sequenced region. Second, cleavage at Lys959 would yield a cross-linked peptide (residues 42−81 of flavodoxin and residues 897−959 of methionine synthase) with a calculated mass of 12 009 Da, and such a peptide was not seen in our analyses (Figures 7 and 8).

To confirm the carboxy-termini of the cross-linked peptides, the LysC digestion samples were analyzed by mass spectrometry (Figure 8). Since the N-termini of the cross-linked peptides are known from sequencing, the mass of the cross-linked species can be used to determine the C-termini. In the cross-linked protein digest, LysC peptide residues 82−131 from flavodoxin and 992−1035 of methionine synthase are seen, establishing that the C-termini of the cross-linked peptides are at or before residue 81 of the flavodoxin peptide and 991 of the methionine synthase peptide. The only lysine between residues 42 and 81 of flavodoxin is Lys81, so residues 42−81 likely comprise the flavodoxin peptide that is cross-linked. The acidic cross-linking residue Glu61, which was identified by sequencing, is contained within this peptide. Six lysines occur between residues 897 and 991 of methionine synthase and could possibly be involved in cross-linking (Lys897, Lys898, Lys959, Lys978, Lys986, and Lys991). Because Lys897 and Lys898 were observed in amino acid sequencing, these are not the cross-linked residues. Cleavage at Lys991 suggests that this also is not a cross-linked lysine. Mass spectral analysis of the digestion mixture reveals two peaks with masses of 14 227 and 14 510 Da. The intensity of these peaks is low, but the observed masses, although outside our expected error of ±14 Da, are consistent with cleavage occurring at Lys978 and Asn981, respectively. They are most consistent with flavodoxin residues 42−81 being cross-linked to methionine synthase residues 897−978 or 897−981 (predicted cross-linked masses of 14 253 and 14 553 Da, respectively). This evidence further supports the involvement of Lys959 of methionine synthase as the basic cross-linking partner to Glu61 of flavodoxin. If Lys959 were not cross-linked, one would expect LysC cleavage at this position rather than the next downstream lysine, residue 978. Lysine 978 and asparagine 981 then represent alternative sites for the next possible LysC cleavage. LysC cleavage at asparagines has previously been reported for other systems (*39*). The remaining LysC peptides 978(981)−986 and 986−991 are too small to detect in the presence of matrix background.

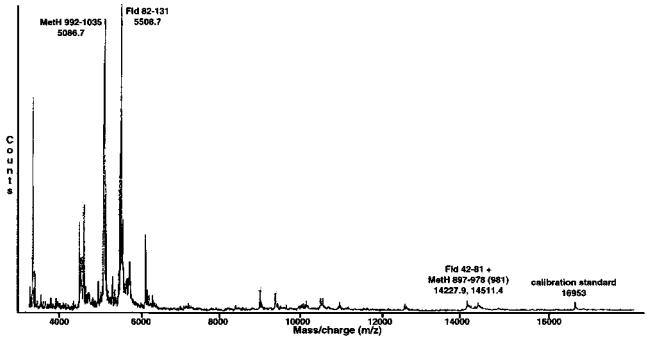

FIGURE 8: Mass spectrum of the LysC digest of the complex between His_6flavodoxin and the 38 kDa activation domain of methionine synthase. Mass analysis assists in identifying the C-termini of the cross-linked peptides. Peaks for flavodoxin LysC residues 82−131 and methionine synthase LysC residues 992−1035 were observed in the digest. Thus, the cross-linked peptide must terminate at or before residue 81 for the flavodoxin moiety and at or before residue 991 for the methionine synthase moiety. The peaks at 14 227.9 and 14 511.4 Da have masses that are consistent with flavodoxin residues 42−81 cross-linking to methionine synthase residues 897−978 and 897−981, respectively.

DISCUSSION

The X-ray structures of *E. coli* flavodoxin (*40*), the cobalamin-binding domain of methionine synthase (*41*), and the activation domain of methionine synthase (*42*) have been determined, and a molecular complex of these proteins has been modeled (*43*). In the model (Figure 9), the cofactors bound to these regions are positioned in proximity for reductive remethylation, i.e., for electron transfer from the FMN of flavodoxin to cobalamin and for subsequent methyl transfer from AdoMet to cobalamin. This model suggests regions involved in interdomain interactions of methionine synthase and in protein−protein interactions between flavodoxin and methionine synthase and predicts extensive contacts between flavodoxin and the 38 kDa activation domain of methionine synthase. The model positions Asp35 and Glu16 of flavodoxin close to Lys1035 of methionine synthase, Glu61 close to Lys959, and Asp65 and Asp11 close to Lys1188. These potential cross-linking residues are located at the periphery of the modeled interface, where steric accommodation of the succinimidyl ester intermediate would be possible without disrupting the complex.

The finding that flavodoxin can be cross-linked to the 38 kDa activation region of methionine synthase is consistent with the model, as is our identification of the cross-link between Glu61 of flavodoxin and Lys959 of methionine synthase. Of the acid−base pairs that are candidates for cross-linking, Glu61 and Lys959 are in closest proximity in the model. The cross-linking and binding experiments also implicate Lys1035 in flavodoxin binding. We observed 43% residual cross-linking level of flavodoxin to the Lys959Thr mutant methionine synthase, which suggests that other cross-links must be present. We did not, however, detect cross-links between Lys1035 and Glu16 or Asp35 since peptides with the expected masses of 8750 or 7745 Da were not observed in the digests of cross-linked wild-type enzyme (Figures 7 and 8). In fact, the presence of the peptide generated by cleavage at Lys1035 (5086 Da) provides evidence that this residue is not cross-linked (Figure 8). It seems likely that other cross-linked peptides were present in the digest but were not identified.

Cross-linking was not observed with the 98 kDa fragment, which contains the substrate-binding and cobalamin-binding regions of methionine synthase but lacks the activation domain (Figure 4, lane 5). This seems puzzling since the model predicts contacts between flavodoxin and the cobalamin-binding domain. One possible explanation for the failure to observe cross-linking between flavodoxin and the 98 kDa domain is that the contact area between flavodoxin and the activation domain is more extensive than that between the cobalamin-binding region and flavodoxin. Thus, in the absence of the activation domain, important binding determinants are missing. An additional explanation is that

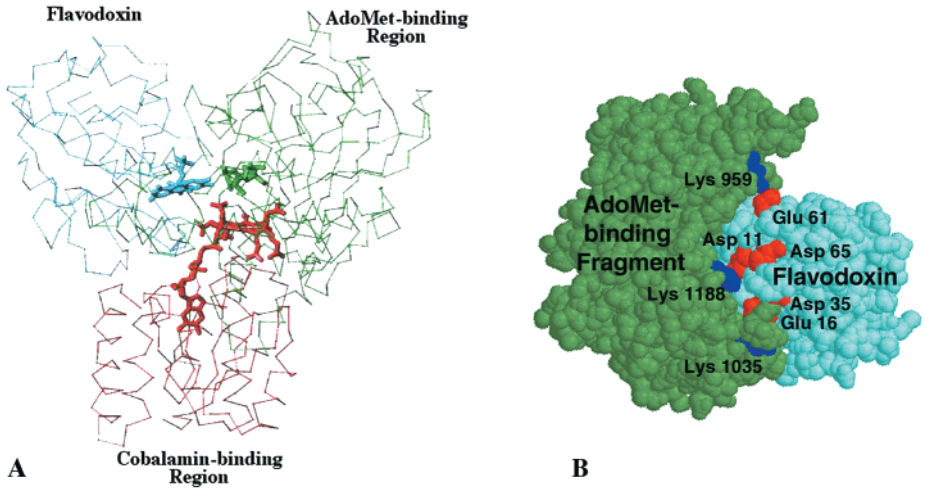

FIGURE 9: Docking simulation of methionine synthase regions and flavodoxin. Identification of candidate cross-linking and binding residues. X-ray structures for flavodoxin and the cobalamin-binding and AdoMet-binding regions of methionine synthase have previously been determined. (A) A model, adapted from ref *43*, positions the cofactors (cobalamin, FMN, and AdoMet) of the cobalamin-binding region (red), flavodoxin (cyan), and 38 kDa activation domain (green) in proximity for electron transfer from FMN to cobalamin and for methyl transfer from AdoMet to cobalamin. This model suggests a binding interface between flavodoxin and the cobalamin-binding and activation modules of methionine synthase. Panel B illustrates a space-filling representation of the docking model after 180° rotation about the *y*-axis and 90° rotation about the *x*-axis. This view reveals several acid−base partners at the edge of the binding interface (Lys959 and Glu61, Lys1188 and Asp65, Lys1188 and Asp11, Lys1035 and Asp35, and Lys1035 and Glu16) between the 38 kDa methionine synthase domain (green) and flavodoxin (cyan).

there are multiple conformational states of methionine synthase, some of which prohibit flavodoxin binding. Our working model is that methionine synthase assumes different conformations during catalytic turnover and activation, and that flavodoxin can form contacts with the cobalamin-binding region only when the methionine synthase holoenzyme is in the activation conformation. In this conformation, the 38 kDa activation domain is positioned with its bound AdoMet above the cobalamin and in proximity to the cobalt. In primary turnover, the cobalamin of methionine synthase reacts readily with homocysteine and methyltetrahydrofolate but not with flavodoxin or AdoMet (*11*). In the reactivation conformation, the cobalamin of methionine synthase reacts with flavodoxin and AdoMet, but not with the primary turnover substrates. Thus, the 98 kDa fragment may be in a turnover conformation, in which the substrate binding region occludes approach of flavodoxin to the cobalamin-binding module.

Identification of the cross-linking and binding residues in the interface between flavodoxin and methionine synthase pinpoints the area of the activation domain that is involved in specific interaction with the flavodoxin electron carrier. The cross-linking experiments thus place strong constraints on the possible arrangements of domains that permit reduction of cob(II)alamin by flavodoxin and methylation by AdoMet. We are currently attempting more complete mapping of the interface between flavodoxin and methionine synthase by structural and other methods. Definition of this interface will provide a foundation for understanding the effects of mutations and polymorphisms observed in the human homologues of flavodoxin and methionine synthase.

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REFERENCES

1. Mayhew, S. G., and Tollin, G. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) pp 389−426, CRC Press, Boca Raton, FL.
2. Smillie, R. M. (1965) *Biochem. Biophys. Res. Commun. 20*, 621−629.
3. Bianchi, V., Eliasson, R., Fontecave, M., Mulliez, E., Hoover, D. M., Matthews, R. G., and Reichard, P. (1993) *Biochem. Biophys. Res. Commun. 197*, 792−797.
4. Ifuku, O., Koga, N., Haze, S., Kishimoto, J., and Wachi, Y. (1994) *Eur. J. Biochem. 224*, 173−178.
5. Vetter, H., Jr., and Knappe, J. (1971) *Hoppe Seyler's Z. Physiol. Chem. 352*, 433−446.
6. Fujii, K., and Huennekens, F. M. (1974) *J. Biol. Chem. 249*, 6745−6753.
7. Drummond, J. T., Huang, S., Blumenthal, R. M., and Matthews, R. G. (1993) *Biochemistry 32*, 9290−9295.
8. Fujii, K., Galivan, J. H., and Huennekens, F. M. (1977) *Arch. Biochem. Biophys. 178*, 662−670.
9. Foster, M. A., Dilworth, M. J., and Woods, D. D. (1964) *Nature 201*, 39−42.
10. Mangum, J. H., and Scrimgeour, K. G. (1962) *Fed. Proc. 21*, 242.
11. Jarrett, J. T., Huang, S., and Matthews, R. G. (1998) *Biochemistry 37*, 5372−5382.
12. Goulding, C. W., Postigo, D., and Matthews, R. G. (1997) *Biochemistry 36*, 8082−8091.
13. Banerjee, R. V., Johnston, N. L., Sobeski, J. K., Datta, P., and Matthews, R. G. (1989) *J. Biol. Chem. 264*, 13888−13895.
14. Gómez-Moreno, C., Sancho, J., Fillat, M., Pueyo, J. J., and Edmondson, D. E. (1987) in *Flavins and Flavoproteins* (Edmondson, D. E., and McCormick, D. B., Eds.) pp 335−339, Walter de Gruyter, Berlin.
15. Dutton, J. E., and Rogers, L. J. (1978) *Biochim. Biophys. Acta 537*, 501−506.
16. Tollin, G., Cheddar, G., Watkins, J. A., Meyer, T. E., and Cusanovich, M. A. (1984) *Biochemistry 23*, 6345−6349.
17. Foust, G. P., Mayhew, S. G., and Massey, V. (1969) *J. Biol. Chem. 244*, 964−970.
18. Hoover, D. M., Jarrett, J. T., Sands, R. H., Dunham, W. R., Ludwig, M. L., and Matthews, R. G. (1997) *Biochemistry 36*, 127−138.
19. Karplus, P. A., Daniels, M. J., and Herriott, J. R. (1991) *Science 251*, 60−66.
20. Ingelman, M., Bianchi, V., and Eklund, H. (1997) *J. Mol. Biol. 268*, 147−157.
21. Leclerc, D., Wilson, A., Dumas, R., Gafuik, C., Song, D., Watkins, D., Heng, H. H., Rommens, J. M., Scherer, S. W., Rosenblatt, D. S., and Gravel, R. A. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 3059−3064.
22. Wilson, A., Leclerc, D., Rosenblatt, D. S., and Gravel, R. A. (1999) *Hum. Mol. Genet. 8*, 2009−2016.
23. Amaratunga, M., Fluhr, K., Jarrett, J. T., Drennan, C. L., Ludwig, M. L., Matthews, R. G., and Scholten, J. D. (1996) *Biochemistry 35*, 2453−2463.
24. Drummond, J. T., Loo, R. R., and Matthews, R. G. (1993) *Biochemistry 32*, 9282−9289.
25. Horton, R. M., Ho, S. N., Pullen, J. K., Hunt, H. D., Cai, Z., and Pease, L. R. (1993) *Methods Enzymol. 217*, 270−279.
26. Fluhr, K. (2000) Ph.D. Dissertation, Department of Biological Chemistry, University of Michigan, Ann Arbor, MI.
27. Drummond, J. T., Jarrett, J., González, J. C., Huang, S., and Matthews, R. G. (1995) *Anal. Biochem. 228*, 323−329.
28. Edmondson, D. E., and Tollin, G. (1971) *Biochemistry 10*, 124−132.
29. Freire, E., Mayorga, O. L., and Straume, M. (1990) *Anal. Biochem. 62*, 950A−959A.
30. Pueyo, J. J., Sancho, J., Edmondson, D. E., and Gómez-Moreno, C. (1989) *Eur. J. Biochem. 183*, 539−544.
31. Pueyo, J. J., Revilla, C., Mayhew, S. G., and Gómez-Moreno, C. (1992) *Arch. Biochem. Biophys. 294*, 367−372.
32. Pirola, M. C., Monti, F., Aliverti, A., and Zanetti, G. (1994) *Arch. Biochem. Biophys. 311*, 480−486.
33. Jung, Y. S., Roberts, V. A., Stout, C. D., and Burgess, B. K. (1999) *J. Biol. Chem. 274*, 2978−2987.
34. McIver, L., Leadbeater, C., Campopiano, D. J., Baxter, R. L., Daff, S. N., Chapman, S. K., and Munro, A. W. (1998) *Eur. J. Biochem. 257*, 577−585.
35. Staros, J. V., Wright, R. W., and Swingle, D. M. (1986) *Anal. Biochem. 156*, 220−222.
36. Cuatrecasas, P., and Parikh, I. (1972) *Biochemistry 11*, 2291−2299.
37. Bordo, D., and Argos, P. (1991) *J. Mol. Biol. 217*, 721−729.
38. Jarrett, J. T., Goulding, C. W., Fluhr, K., Huang, S., and Matthews, R. G. (1997) *Methods Enzymol. 281*, 196−213.
39. Sweeney, P. J., and Walker, J. M. (1993) in *Methods in Molecular Biology, Vol. 16: enzymes of molecular biology* (Burrell, M. M., Ed.) pp 277−303, Humana Press, Totowa, NJ.
40. Hoover, D. M., and Ludwig, M. L. (1997) *Protein Sci. 6*, 2525−2537.
41. Drennan, C. L., Huang, S., Drummond, J. T., Matthews, R. G., and Ludwig, M. L. (1994) *Science 266*, 1669−1674.
42. Dixon, M. M., Huang, S., Matthews, R. G., and Ludwig, M. (1996) *Structure 4*, 1263−1275.
43. Drennan, C. L., Dixon, M. M., Hoover, D. M., Jarrett, J. T., Goulding, C. W., Matthews, R. G., and Ludwig, M. L. (1998) in *Vitamin B12 and B12-Proteins* (Kraüler, B., Arigoni, D., and Golding, B. T., Eds.) pp 133−155, Wiley-VCH, Weinheim, Germany.

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