Differences in the Efficiency of Reductive Activation of Methionine Synthase and Exogenous Electron Acceptors between the Common Polymorphic Variants of Human Methionine Synthase Reductase†

Horatiu Olteanu, Troy Munson, and Ruma Banerjee*

Biochemistry Department, University of Nebraska, Lincoln, Nebraska 68588-0664

Received August 16, 2002

ABSTRACT: Methionine synthase reductase (MSR) catalyzes the conversion of the inactive form of human methionine synthase to the active state of the enzyme. This reaction is of paramount physiological importance since methionine synthase is an essential enzyme that plays a key role in the methionine and folate cycles. A common polymorphism in human MSR has been identified (66A → G) that leads to replacement of isoleucine with methionine at residue 22 and has an allele frequency of 0.5. Another polymorphism is 524C → T, which leads to the substitution of serine 175 with leucine, but its allele frequency is not known. The I22M polymorphism is a genetic determinant for mild hyperhomocysteinemia, a risk factor for cardiovascular disease. In this study, we have examined the kinetic properties of the M22/S175 and I22/S175 and the I22/L175 and I22/S175 pairs of variants. EPR spectra of the semiquinone forms of variants I22/S175 and M22/S175 are indistinguishable and exhibit an isotropic signal at $g = 2.00$. In addition, the electronic absorption and reduction stoichiometries with NADPH are identical in these variants. Significantly, the variants activate methionine synthase with the same V_{max}; however, a 3−4-fold higher ratio of MSR to methionine synthase is required to elicit maximal activity with the M22/S175 and I22/L175 variant versus the I22/S175 enzyme. Differences are also observed between the variants in the efficacies of reduction of the artificial electron acceptors: ferricyanide, 2,6-dichloroindophenol, 3-acetylpyridine adenine dinucleotide phosphate, menadione, and the anticancer drug doxorubicin. These results reveal differences in the interactions between the natural and artificial electron acceptors and MSR variants in vitro, which are predicted to result in less efficient reductive repair of methionine synthase in vivo.

Methionine synthase reductase (MSR,[1] EC 2.1.1.135) is a 78 kDa dual flavoprotein involved in the reductive activation of methionine synthase (*1*). It contains stoichiometric amounts of FAD and FMN, which are postulated to transfer reducing equivalents sequentially from the two-electron donor, NADPH, to the one-electron acceptor, the cob(II)alamin cofactor of methionine synthase (Figure 1). MSR is also capable of reducing cytochrome *c* in vitro. The sequence of MSR is homologous with those of other eukaryotic (cytochrome P450 oxidoreductase, nitric oxide synthase, and NR1) and prokaryotic (sulfite reductase and cytochrome P450 BM3) diflavin enzymes. The primary structure of the prototype of this family of diflavin reductases, cytochrome P450 oxidoreductase, predicts the existence of two domains: an N-terminal flavodoxin-like FMN binding domain and a C-terminal flavodoxin reductase-like domain,

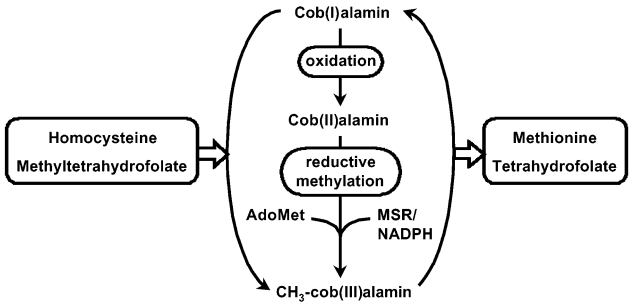

FIGURE 1: Catalytic cycle and activation mechanism of methionine synthase.

which binds FAD and NADPH (*2*). The crystal structure of recombinant rat liver cytochrome P450 oxidoreductase confirms this modular organization, showing the FMN domain connected via a flexible linker to the FAD/NADPH domain (*3*). The linker allows spatial proximity of the two flavin cofactors, permitting them to move to within 4 Å of each other for direct electron transfer.

The physiological function of MSR is important for the sustained activity of methionine synthase, a central enzyme in homocysteine and folate metabolism. Hereditary defects leading to functional methionine synthase deficiency are inherited as autosomal recessive disorders (*4*). These patients belong to one of two complementation groups, *cbl*G and

† This research was supported by National Institutes of Health Grant DK45776 (to R.B.) and a Predoctoral Fellowship from the Heartland Affiliate of the American Heart Association (to H.O.). R.B. is an Established Investigator of the American Heart Association.

* To whom correspondence should be addressed. Telephone: (402) 472-2941. Fax: (402) 472-7842. E-mail: rbanerjee1@unl.edu.

[1] Abbreviations: MSR, human methionine synthase reductase; DCIP, 2,6-dichloroindophenol; 3-AcPy ADP, 3-acetylpyridine adenine dinucleotide phosphate; AdoMet, *S*-adenosylmethionine; CH_3-H_4folate, 5-methyl tetrahydrofolate; GST, glutathione *S*-transferase; NR1, novel reductase 1; SEM, standard error of the mean.

10.1021/bi020536s CCC: $22.00
Published on Web 10/18/2002

*cbl*E, with mutations in methionine synthase and MSR, respectively. Both classes of inborn errors lead to severe hyperhomocysteinemia, hypomethionemia, and megaloblastic anemia without methylmalonic aciduria (*4*). Mutations distributed along the entire coding region of the MSR gene have been identified (*5*, *6*), and these patients exhibit a range of clinical symptoms, including developmental delay, ataxia, cerebral atrophy, neonatal seizures, and blindness, as well as thrombosis and arteriosclerotic vascular modifications (*7*, *8*).

Results from numerous epidemiological studies have led to the identification of elevated levels of plasma homocysteine as a risk factor for cardiovascular diseases, neural tube defects, and Alzheimer's disease (*9*−*13*). This has stimulated a high level of interest in identifying common polymorphisms in genes involved in homocysteine metabolism and assessing their association, if any, with mild hyperhomocysteinemia and an increased risk for cardiovascular diseases and neural tube defects. The most extensively studied polymorphism to date in this pathway is the 677C → T variation that leads to a thermolabile form of methylenetetrahydrofolate reductase (*14*). Recently, a common polymorphism in the MSR gene, 66A → G, has been described which has an allele frequency of ∼0.51 in the North American population (*15*). This leads to a change in the coding sequence from isoleucine to methionine at position 22 and is reported to be correlated with an increased risk for premature coronary artery disease (*16*), spina bifida (*15*), and Down's syndrome (*17*, *18*). In addition, this polymorphism is reported to be a genetic determinant of elevated plasma homocysteine levels (*19*), and 66AA homozygous males (29% in the Northern-Irish population) were found to have a 4% increase in cardiovascular risk as compared to 66GG homozygotes (*19*).

Besides this relatively well-studied polymorphism, a second MSR variant containing leucine instead of serine at position 175 came to our attention during our cloning experiments, and its presence was confirmed by a separate entry in the gene database (NCBI Annotation Project GenBank entry XP_003862). This polymorphism occurs at position 524 in the MSR coding sequence, and to our knowledge, no genetic or population data have been reported on it.

In the study presented here, we compare the kinetic and spectroscopic properties of the I22/S175 enzyme, which we have characterized previously, with the M22/S175 and I22/L175 variants to assess the penalties, if any, that are associated with these polymorphisms. Although the flavin content and the spectroscopic properties of the three variants are indistinguishable, they clearly differ in their efficacies of reduction of the natural electron acceptor, methionine synthase, and a number of unnatural electron acceptors. These results provide important insights into how the common polymorphism, I22M, could influence methionine synthase activation under physiological conditions and reveal a biochemical phenotype for the previously undescribed S175L polymorphism.

MATERIALS AND METHODS

Chemicals and Reagents. Chemicals were purchased from Sigma (St. Louis, MO) and enzymes from Life Technologies (Rockville, MD) and Fermentas (Hanover, MD). Oligonucleotides were purchased from Sigma Genosys and Integrated DNA Technologies (Coralville, IA). Glutathione Sepharose 4B, Sephacryl S200, and (6-*R*,*S*)-5-[$^{14}CH_3$]-H_4folate (barium salt, 55 mCi/mmol) were purchased from Amersham Pharmacia (Piscataway, NJ). (6-*R*,*S*)-CH_3-H_4folate (calcium salt) was obtained from Dr. Schirck's laboratory (Switzerland). All solvents were HPLC grade. Porcine livers were obtained fresh from a local slaughterhouse, cubed, and stored frozen at −80 °C until further use.

Generation of the Polymorphic Variants of Human Methionine Synthase Reductase. Site-directed mutations were engineered in the MSR expression vector pHOMSR (*1*), using the QuickChange mutagenesis kit from Stratagene. The parent plasmid contained isoleucine and serine at positions 22 and 175, respectively. Forward (5′-GGCATCACCTGCATCCTTGAGGACAGACCTTGTG-3′) and reverse (5′-CACAAGGTCTGTCCTCAAGGATGCAGGTGATGCC-3′) primers were designed to generate the first polymorphism (I22/L175), by replacing serine 175 with leucine. A *Bgl*II−*Kpn*I fragment containing this mutation was exchanged with the corresponding fragment in the parent plasmid. The presence of the mutation is accompanied by the loss of an *Xho*I restriction site and was initially verified by restriction enzyme digestion and confirmed thereafter by DNA sequencing.

The M22/S175 variant was generated using a similar strategy, using the following mutagenic primers [5′-CCATCGCAGAAGAAATGTGTGAGCAAGCTGTGG-3′ (forward) and 5′-CCACAGCTTGCTCACACATTTCTTCTGCGATGG-3′ (reverse)]. A *Csp*45I−*Kpn*I fragment was exchanged with the corresponding fragment in the parent plasmid. The introduction of the M22 mutation in the resulting M22/S175 clone was verified by DNA sequencing.

Expression and Purification of Human Methionine Synthase Reductase Variants and Porcine Methionine Synthase. The MSR polymorphic variants, M22/S175 and I22/L175, were expressed as GST fusion proteins in *Escherichia coli* strain BL21(DE3), using the protocol described for the parent clone (*1*). The GST tag was removed during purification by limited proteolysis as described previously (*1*). Approximately 1.5 mg of MSR M22/S175 and 2 mg of I22/L175 were obtained per liter of culture. Porcine methionine synthase was purified as described previously (*20*, *21*).

Flavin Cofactor Determination. The flavin cofactor content was determined by HPLC (*22*) using a fluorescence detector, as described previously (*1*).

UV−*Visible Spectroscopic Analysis of MSR Polymorphisms*. Redox titrations were performed in 50 mM Tris buffer (pH 7.5) containing 10% (v/v) glycerol at room temperature in an anaerobic chamber with a 95/5 N_2/H_2 atmosphere (oxygen concentration of <5 ppm). Protein samples and titration reagents were made anaerobic by flushing them with oxygen-free nitrogen. Enzyme solutions were then titrated using aliquots of the NADPH solution as the reductant, using concentrations that are described in the figure legends.

EPR Spectroscopy. Enzyme reduced with 1 equiv of NADPH under aerobic or anaerobic conditions was transferred to 4 mm quartz tubes and frozen in liquid nitrogen. EPR spectra were recorded at 77 K in a Bruker ESP 300E spectrometer equipped with a Hewlett-Packard microwave frequency counter (model 5253B) and a Bruker gaussmeter

(model ER 035). The spin concentration of the semiquinone radical was quantified by double integration of the spectra and comparison to a sample of 1 mM Cu(II)perchlorate, using the Aasa-Vänngård correction for the *g* factor (*23*). Power saturation of the signals was determined by analyzing the dependence of the signal intensity on the microwave power according to eq 1

$$I = I_0[P/(1 + P/P_{1/2})^b]^{0.5} \quad (1)$$

where I represents the signal intensity at a given power P, I_0 represents the amplitude, and b is the homogeneity parameter (*24*). Line widths were measured between the extrema of the first derivatives of the recorded spectra.

Enzyme Assays. Enzyme activity was monitored in two types of assays: methionine synthase activation and reduction of artificial electron acceptors. The activity of MSR in methionine synthase reactivation was performed as described previously (*1*). The cytochrome *c* reductase activity was determined as described previously (*1*) except that 50 mM Tris (pH 7.5) was employed as the buffer and the rates were determined at 37 °C. The reaction mixture for the assay (total volume of 1 mL) with other electron acceptors contained 50 mM Tris buffer (pH 7.5), 100 μM NADPH, MSR, and varying concentrations of the electron acceptor. The reaction was initiated by addition of enzyme, and the reaction rate was monitored spectrophotometrically at 37 °C. The following extinction coefficients were used to calculate the rates of reduction of the individual substrates: 21 mM^{-1} cm^{-1} at 600 nm for DCIP (*25*) and 5.6 mM^{-1} cm^{-1} at 363 nm for 3-AcPy ADP (*26*). Reduction of ferricyanide, menadione, and doxorubicin were assessed indirectly by following NADPH oxidation at 340 nm. Background rates in the absence of enzyme were subtracted from the rates recorded in the presence of enzyme.

RESULTS

Expression and Purification of Polymorphic Variants of MSR. The M22/S175 and I22/L175 variants were expressed as GST fusion proteins and readily purified using a combination of affinity chromatography, thrombin cleavage, and anion exchange chromatography steps employed previously for the I22/S175 variant. The I22M polymorphism is located in the putative FMN binding domain, while the S175L polymorphism is close to the C-terminus of the same domain, in the predicted linker region between the FMN and FAD/NADPH binding domains. The variant proteins were purified to >90% homogeneity as determined by SDS−PAGE (Figure 1 of the Supporting Information). The chromatographic behavior of the M22/S175 and I22/L175 variants was identical to that of the previously described I22/S175 protein, suggesting that major conformational changes are not induced by the mutations.

Characterization of the Flavin Content of Methionine Synthase Reductase Variants. HPLC analysis of heat-denatured enzyme samples revealed the presence of FMN and FAD, two fluorophores whose retention times exactly matched those of commercially available standards. Quantitative evaluation of the peak areas showed that the FAD content and the FMN content of purified MSR M22/S175 were 0.96 ± 0.04 and 0.97 ± 0.05 mol/mol of enzyme (mean values ± SEM, $n = 3$), respectively. For the I22/L175 variant, the FAD content and the FMN content were 0.96 ± 0.02 and 0.91 ± 0.03 mol/mol of enzyme (mean values ± SEM, $n = 3$), respectively.

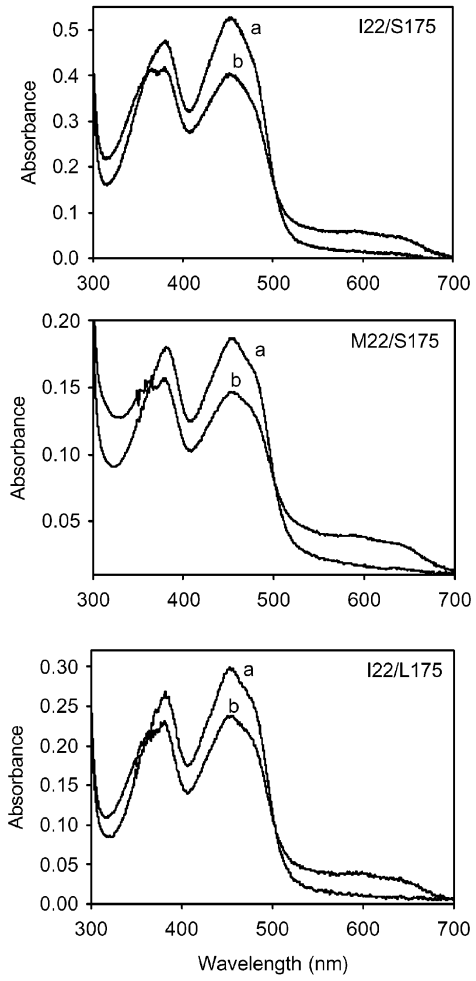

FIGURE 2: UV−visible absorbance spectra of the recombinant I22/S175, M22/S175, and I22/L175 MSR variants. In each panel, the spectra of the oxidized (a) and reduced semiquinone (b) form of the enzyme [25, 9, and 14 μM in the top, middle and bottom panels, respectively, in 50 mM Tris buffer (pH 7.5)] are shown. The reduced semiquinone was produced following the addition of a 5-fold molar excess of NADPH under aerobic conditions.

UV−Visible Spectra of Methionine Synthase Reductase. The overall spectral characteristics of the M22/S175 and I22/L175 MSR variants were similar to those of the I22/S175 protein (Figure 2). The oxidized enzymes had absorption maxima at 380 and 454 nm. Reduction of oxidized MSR I22/S175 under aerobic conditions led to the appearance of an air-stable semiquinone with a broad long-wavelength absorption centered at 580 nm (*1*). A stoichiometry of 1 mol of NADPH/mol of enzyme was observed for maximal semiquinone formation during aerobic titration of the I22/S175 variant (Figure 3A). The I22/S175 and M22/L175 variants displayed identical behavior (data not shown). When the titrations of the I22/S175 and M22/L175 variants were performed anaerobically, reduction was observed to proceed in two stages (Figure 3B,C). In the first stage, formation of the blue neutral semiquinone with an absorption maximum of 580 nm was seen. The maximal concentration of semiquinone was observed at a stoichiometry of 1 mol of NADPH/mol of enzyme (Figure 3B). In the second stage, complete reduction of the enzyme was observed at a

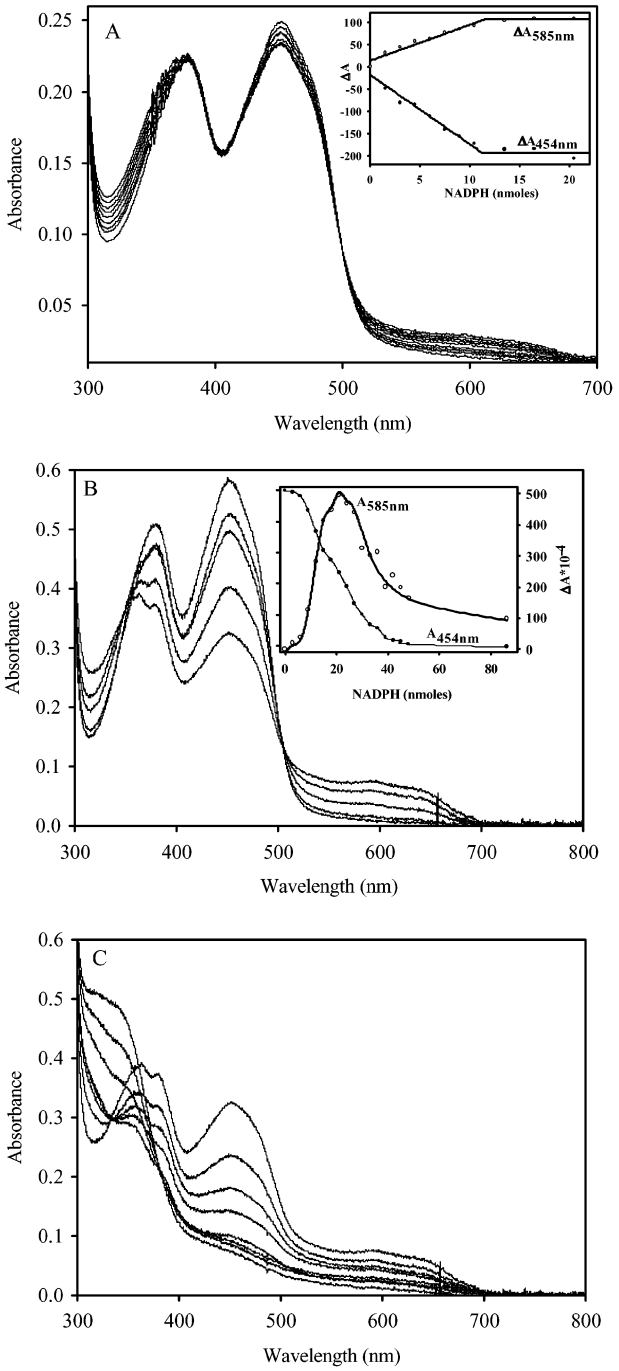

FIGURE 3: Reduction of oxidized I22/S175 MSR with NADPH under aerobic and anaerobic conditions. (A) A sample of 13 μM MSR (23 nmol of flavin) in 50 mM Tris buffer (pH 7.5) was reduced aerobically with a stock solution of 1 mM NADPH. Spectra were recorded after incremental addition of 1 μL (1 nmol) aliquots of NADPH. The inset shows a plot of absorbance changes as a function of the amount of reducing agent added. (B) A sample of 30 μM MSR (43 nmol of flavin) in 50 mM Tris buffer (pH 7.5) was reduced anaerobically with a stock solution of 1 mM NADPH. Spectra were recorded after incremental addition of 3 μL (3 nmol) aliquots of NADPH, up to a total amount of 25 nmol. (C) Spectra recorded for the same sample as described for panel B, upon incremental addition of NADPH to a total amount of 80 nmol. The inset in panel B shows a plot of absorbance changes as a function of the amount of NADPH added.

stoichiometry of 2 mol of NADPH/mol of enzyme as indicated by bleaching of the flavin absorption peaks at 380 and 454 nm (Figure 3C).

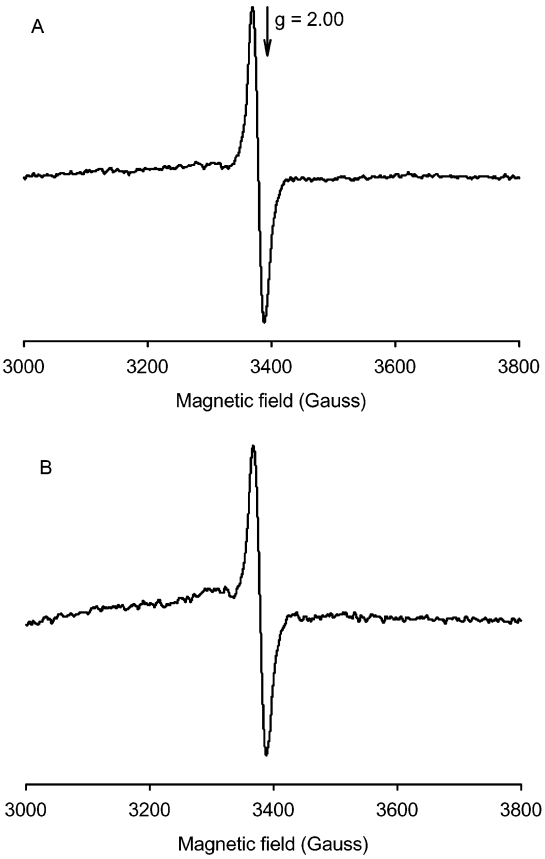

FIGURE 4: EPR spectra of reduced I22/S175 MSR. (A) MSR (25 μM) in 50 mM Tris buffer (pH 7.5) was reduced aerobically with 100 μM NADPH. EPR conditions were as follows: temperature, 77 K; microwave frequency, 9.466 GHz; microwave power, 20 μW; gain, 20 000; modulation frequency, 100 kHz; and modulation amplitude, 4.039 G. (B) MSR (26 μM) in 50 mM Tris (pH 7.5) was reduced anaerobically with 26 μM NADPH. EPR conditions were as described for panel A.

Characterization of the Flavin Semiquinone Radical by EPR Spectroscopy. The semiquinones formed during reduction under aerobic and anaerobic conditions were characterized by EPR spectroscopy, and the concentration of the organic radical was determined (Figure 4). In the absence of NADPH, an EPR signal was not observed. In the presence of NADPH, an isotropic $g = 2.0$ signal characteristic of an organic semiquinone radical was seen. The EPR spectra of both the blue neutral and red anionic semiquinone species have the same g value (*27*). However, they can be qualitatively distinguished by differences in their line widths (*27*), i.e., the distance between the positive and negative extrema in the first derivative of the recorded spectra (*28*, *29*). The neutral semiquinone is characterized by a line width of 19 G, while the anionic semiquinone signal is characterized by a line width of 15 G. Intermediate values suggest a mixture of the two types of the semiquinone. The EPR spectral line width of all three MSR variants was 20 G, consistent with the presence of a blue neutral semiquinone. The microwave power saturation dependence of the EPR signal yielded a $P_{1/2}$ of 25 μW.

Spin concentrations of 0.8 and 1.8 spins/mol of enzyme were observed for samples reduced under aerobic and anaerobic conditions, respectively. This is consistent with the presence of ~1 equiv of semiquinone/mol of enzyme under aerobic conditions and ~2 equiv of semiquinone/mol

Table 1: Steady State Kinetic Parameters for Methionine Synthase Activation by MSR Polymorphic Variants

variant	V_{max} (μmol min^{-1} mg^{-1})	MSR/MS for V_{max}
I22/S175[a]	2.2 ± 0.1	3.1 ± 0.5
M22/S175	2.3 ± 0.1	12.4 ± 1.4
I22/L175	2.4 ± 0.1	10.3 ± 0.9

[a] Results taken from ref *1*. MS denotes methionine synthase.

Table 2: Comparison of Reductase Activity with Artificial Electron Acceptors in Members of the Dual Flavoprotein Oxidoreductase Family

	k_{cat} (s^{-1})				
enzyme	ferricyanide	DCIP	3-AcPy ADP	menadione	doxorubicin
MSR I22/S175	8.24	0.43	0.86	1.61	1.61
P450 reductase	164.8[a]	32.67[a]	2.02[a]	41.9[b]	15.4[b]
NR1[c]	4.5	ND[e]	ND[e]	0.9	0.6
NOS III reductase[d]	ND[e]	ND[e]	ND[e]	2.07	1.45

[a] Rat liver cytochrome P450 reductase (results taken from ref *30*). [b] Human recombinant cytochrome P450 reductase (results taken from ref *28*). [c] Results taken from ref *28*. [d] Reductase domain of bovine nitric oxide synthase III (results taken from ref *29*). [e] Not determined.

of enzyme under anaerobic conditions as seen by electronic absorption spectroscopy (Figure 3).

Methionine Synthase Reductase Activity. The M22/S175 and I22/L175 variants reactivate methionine synthase activity in a saturable manner, as seen previously with the I22/S175 protein. Activation of methionine synthase by MSR exhibits an ionic strength dependence with a maximum at ~220 mM (*1*), which is similar in all three variants (data not shown). A comparison of the Michaelis−Menten parameters for the activation of methionine synthase reveals that V_{max} is unaffected by the polymorphisms (Table 1). However, 4- and 3-fold higher molar ratios of MSR to methionine synthase are required for maximal activation with the M22/S175 and I22/L175 variants, respectively, as compared to that of the I22/S175 enzyme.

Relative Efficacies of MSR Variants in Electron Transfer to Artificial Electron Acceptors. The cytochrome *c* reductase activities of the M22/S175 (1297 nmol mg^{-1} min^{-1}) and I22/L175 (1297 nmol mg^{-1} min^{-1}) variants were ~1.5-fold lower than that of the I22/S175 variant (1893 nmol mg^{-1} min^{-1}). The kinetic parameters for the reduction of other one- and two-electron acceptors by I22/S175 MSR are compared to the activities of other dual flavoprotein oxidoreductases, viz., cytochrome P450 reductase, NR1, and nitric oxide synthase, in Table 2. In addition to the commonly used artificial acceptors, viz., potassium ferricyanide, DCIP, and 3-AcPy ADP, we have also examined the reduction of the anticancer drug doxorubicin and the oxidative stress inducing agent menadione. The activity of I22/S175 MSR is comparable to those of NR1 (*30*) and the reductase domain of bovine nitric oxide synthase III (*31*) but lower than that of cytochrome P450 reductase (*30*−*32*). The reduction of artificial electron acceptors by the M22/S175 and I22/S175 variants is compared in Table 3. In each case, the K_ms for the substrate are similar, but the V_{max} of the M22/S175 variant is ~1.4-fold lower than that for the I22/S175 enzyme.

Table 3: Comparison of the Kinetic Parameters for Reduction of Artifical Electron Acceptors by MSR Variants

	MSR I22/S175		MSR M22/S175	
substrate	V_{max}[a]	K_m (μM)	V_{max}[a]	K_m (μM)
ferricyanide	5440 ± 182	774 ± 53	3819 ± 226	663 ± 87
DCIP	515 ± 32	3.8 ± 0.8	346 ± 13	2.3 ± 0.5
3-AcPy ADP	661 ± 30	18 ± 1.5	866 ± 12	17.7 ± 0.8
menadione	1241 ± 30	18 ± 1.5	866 ± 12	17.7 ± 0.8
doxorubicin	1233 ± 45	28.6 ± 3.3	853 ± 35	36.6 ± 4.4

[a] V_{max} is expressed in units of nanomoles per milligram per minute at 37 °C.

DISCUSSION

The biochemical properties of a common polymorphism (I22M) and a second one (S175L) in human MSR have been examined in this study. The I22M polymorphism in MSR has a high allele frequency of ~0.55 for the M22 allele in the populations studied so far (*15*, *19*, *33*), and it appears to represent a risk factor for premature cardiovascular disease (*16*), neural tube defects (*15*), and Down's syndrome (*17*, *18*). The high prevalence of this MSR polymorphism underscores a critical need to assess the biochemical phenotypes associated with the products of the two alleles. The location of the I22M polymorphism in the predicted FMN domain suggests that it may influence the interaction between MSR and its redox partner, methionine synthase. In this study, we have focused primarily on comparing the kinetic and spectroscopic properties of the I22M variant in addition to characterizing a second polymorphism, S175L.

The high allele frequency of the I22M polymorphism renders the task of assigning the parental allele difficult. However, sequence comparison of MSR and other dual flavoproteins, viz., flavodoxins, cytochrome P450 reductases, nitric oxide synthases, and NR1, reveals that the residue at the equivalent position is either isoleucine, leucine, or valine (Figure 5). Methionine is not seen in proteins in this family at the homologous position (*15*). On the basis of this analysis, the I22 genotype is assigned as the "wild type" and M22 as the polymorphic variant.

The I22M variation is located in the FMN domain, as predicted by the sequence comparison with P450 reductase and related proteins (*3*). In analogy to the bacterial system in which the FMN-containing flavodoxin serves as the proximal electron donor of methionine synthase, the FMN domain of MSR is predicted to interact with human methionine synthase (*34*, *35*). Methionine synthase undergoes a significant structural reorganization during the transition from the catalytically active to inactive state, which is effected by major domain movements (*36*). Residue I22 in *E. coli* flavodoxin is the homologue of I22 in human MSR and, though only partially surface exposed, is located at the edge of the surface that binds bacterial methionine synthase (Figure 6). In fact, the adjoining residues (K20, M21, and K24) contribute to the binding surface that interacts with the AdoMet domain of *E. coli* methionine synthase (*35*). This suggests that an I → M substitution at this position could influence the interaction between the two redox partners and consequently impact methionine synthase activation (Figure 6). The effect of this polymorphism on the affinity for FMN has not been determined directly; however, both the I22/S175 and M22/S175 variants are isolated with 1/1 ratios of FMN or FAD per mole of MSR. This issue is germane because riboflavin, the precursor of FMN and FAD cofactors, is an independent genetic determinant of plasma homocys-

```
MSR_HUMAN........--MRR----FLLLYATQQGQAKAIAEEICEQAVVHGFSADLHCIS--ESD
FLAV_ECOLI.......--AITG-----IFFGSDTGNTENIAKMIQKQLGK--DVADVHDIA--KSS
CPR_HUMAN.......EKMKKTGRNIIVFYGSQTGTAEEFANRLSKDAHR--YGMRGMSADPEEYD
NOS1_HUMAN......GQAMAKRVKATILYATETGKSQAYAKTLCEIFKH---AFDAKVMSMEEYD
NR1_HUMAN........--MPSP--QLLVLFGSQTGTAQDVSERLGREARRRRLGCRVQALD--SYP
```

FIGURE 5: Amino acid sequence comparison of human MSR (MSR_HUMAN, SwissProt accession number Q9UBK8-01), *E. coli* flavodoxin (FLAV_ECOLI, SwissProt accession number P23243), human cytochrome P450 reductase (CPR_HUMAN, SwissProt accession number P16435), human neuronal nitric oxide synthase (NOS1_HUMAN, SwissProt accession number P29475), and human NR1 (NR1-HUMAN, SwissProt accession number Q9UHB4). Residues corresponding to isoleucine 22 in MSR are highlighted in the gray box.

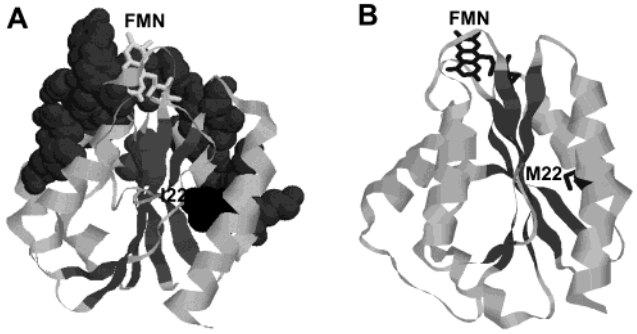

FIGURE 6: Location of isoleucine 22 in flavodoxin and in the modeled structure of the FMN binding domain of human MSR. (A) Structure of bacterial flavodoxin (PDB entry 1AHN) showing FMN in stick display. The surface residues that interact with the AdoMet binding domain of methionine synthase as described in refs *34* and *35* are shown in space filling representation, and the location of residue I22 is indicated. (B) Modeled structure of the M22 variant of the FMN binding domain of human MSR, showing the location of the polymorphic residue and of the cofactor in stick display. The model was generated using the automated homology modeling server Swiss-Model (*49*).

teine (*37*). Interestingly, flavin dissociation from methylenetetrahydrofolate reductase containing the 677C → T polymorphism, which displays a lower affinity for the cofactor, is thought to lead to the higher homocysteine levels associated with this genotype (*38*).

The high prevalence of the I22M variation suggests that differences in the biochemical phenotypes of each polymorphism will be subtle rather than catastrophic. To assess the biochemical consequences of this polymorphism, we have compared the spectroscopic and kinetic properties of the variants. Both the I22/S175 (Figure 3) and M22/S175 (data not shown) variants of MSR exhibit identical electronic absorption changes on reduction with NADPH under aerobic or anaerobic conditions. Under aerobic conditions, formation of the semiquinone was maximal at a stoichiometry of 1 mol of NADPH/mol of enzyme. Under anaerobic conditions, maximal semiquinone formation was also observed at a stoichiometry of 1 mol of NADPH/mol of enzyme and was followed by complete flavin reduction at a stoichiometry of 2 mol of NADPH/mol of enzyme.

Yeast, *Arabidopsis* ATR1 (*39*), mammalian cytochrome P450 reductases (*40*, *41*), and neuronal nitric oxide synthase (*42*) are isolated with an air-stable blue semiquinone. In these proteins, the FMN hydroquinone is the electron donor and the FMN semiquinone has the highest redox potential (*41*). These enzymes cycle between the one-, three-, two-, and one-electron-reduced states during catalysis (*41*). In contrast, the flavins in the bacterial P450 BM3 from *Bacillus megaterium*, which is a fusion between a cytochrome P450 and cytochrome P450 reductase, are completely oxidized as isolated (*43*). Reduction of P450 BM3 with 1 equiv of NADPH under anaerobic conditions does not lead to the formation of semiquinone intermediates, and the protein cycles between the 0, 2, 1, and 0 reduction states during catalysis. The FMN semiquinone serves as the electron donor to heme, and the FMN hydroquinone has the highest redox potential (*41*, *43*).

The three MSR variants are isolated with the flavins in the completely oxidized state rather than in the semiquinone state. Despite this, MSR resembles the eukaryotic rather than the prokaryotic reductases described above. Thus, semiquinone intermediates are formed in the presence of 1 equiv of NADPH under both aerobic and anaerobic conditions as seen by electronic absorption (Figure 3) and EPR (Figure 4) spectroscopy. In contrast, flavin hydroquinone is formed in bacterial P450 BM3 in the presence of 1 equiv of NADPH. Understanding the pathway for electron flow in human MSR awaits detailed analysis of the potentials of each redox couple for the two flavin cofactors. However, unlike the other reductases discussed above, which transfer electrons in two one-electron steps to their physiological partners, human MSR presumably transfers a single electron to the cob(II)alamin in methionine synthase in each reductive repair reaction. This raises the potentially important issue of how the enzyme discharges the second electron, particularly if the resting form is not a one-electron-reduced species. Uncoupled oxygen activation by nitric oxide synthase generates superoxide anion and is observed in the absence of arginine (*44*).

Activation of methionine synthase by the three variants revealed that a 3−4-fold higher ratio of MSR to methionine synthase is necessary with the I22/L175 and M22/S175 variants to achieve the same V_{max} that is seen for the I22/S175 enzyme. The ionic strength dependence of methionine synthase activation is unaffected by these substitutions. Thus, the steady state kinetic analysis indicates a significantly decreased affinity for methionine synthase accompanying substitution of I22 with M22 and S175 with L175. The unavailability of recombinant mammalian methionine synthase precludes direct assessment of the binding affinities between the redox partners as has been done with the bacterial enzymes (*35*, *45*) because the wild-type enzyme can be purified in only very small quantities (*46*). The 3−4-fold difference in the affinities in the MSR variants for methionine synthase suggested by the steady state kinetic analysis seems to be large for a polymorphism with high prevalence. However, it is important to note that methionine synthase reactivation is necessary occasionally rather than in every turnover cycle. In bacteria, it is estimated that methionine synthase leaks out of the catalytic cycle once every 2000 turnovers (*47*, *48*). Thus, Nature is able probably

to tolerate the 4-fold difference between the polymorphic variants because of the occasional need for the activation system.

The ability of MSR to reduce artificial electron acceptors is interesting because some of these are biologically important, viz., activation of the chemotherapeutic agent doxorubicin. Comparison of the cytochrome *c* reductase activity of the I22/S175 enzyme to that of the related flavoenzymes, P450 reductase and NR1, has been reported previously (*1*). In this study, we have examined the activity of the MSR variants, I22/S175 and M22/S175, with a set of artificial electron acceptors (Table 3) and compared the catalytic properties of MSR I22/S175 with those of other diflavoenzymes (Table 1). The I22/S175 and M22/S175 variants exhibit the same V_{max} values. However, the K_m for the M22/S175 variant is 1.4-fold lower with all the acceptors that were examined than that of the I22/S175 enzyme.

The difference in the efficiencies of the I22/S175 and M22/S175 variants in activating methionine synthase may explain the elevated homocysteine level reported in subjects who are homozygous for the M22 polymorphism (*19*). Other interacting factors such as nutritional, specifically vitamin, status are also likely to contribute to hyperhomocysteinemia. As noted in a recent study, compound heterozygosity for multiple polymorphic alleles of MSR and methylenetetrahydrofolate reductase may play a role in the etiology of birth defects and cardiovascular disease (*33*).

In addition to the polymorphic variation at residue 22, we have characterized the variation at residue 175. This polymorphism is identified from comparison of MSR sequences in the database, but its prevalence is unknown. The I22/L175 variant required a 3-fold higher ratio of MSR to methionine synthase to achieve full activation as compared to the I22/L175 variant. Thus, the phenotypic manifestation of this variant is also in the interaction between the redox partners, MSR and methionine synthase.

In summary, this study reveals a significant difference in the relative efficacies of the very common polymorphic variant of MSR, I22M, and suggests a molecular mechanism underlying the risk associated with the M22 allele for mild hyperhomocysteinemia.

SUPPORTING INFORMATION AVAILABLE

Variant proteins purified to >90% homogeneity as determined by SDS−PAGE. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Olteanu, H., and Banerjee, R. (2001) *J. Biol. Chem. 276*, 35558−35563.
2. Porter, T. D., and Kasper, C. B. (1986) *Biochemistry 25*, 1682−1687.
3. Wang, M., Roberts, D. L., Paschke, R., Shea, T. M., Masters, B. S. S., and Kim, J.-J. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 8411−8416.
4. Watkins, D., and Rosenblatt, D. S. (1989) *Am. J. Med. Genet. 34*, 427−434.
5. Leclerc, D., Wilson, A., Dumas, R., Gafuik, C., Song, D., Watkins, D., Heng, H. H. Q., Rommens, J. M., Scherer, S. W., Rosenblatt, D. S., and Gravel, R. A. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 3059−3064.
6. Wilson, A., Leclerc, D., Rosenblatt, D. S., and Gravel, R. A. (1999) *Hum. Mol. Genet.*, 2009−2016.
7. Watkins, D., and Rosenblatt, D. S. (1988) *J. Clin. Invest. 81*, 1690−1694.
8. Steen, C., Rosenblatt, D. S., Scheying, H., Braeuer, H. C., and Kohlschutter, A. (1997) *J. Inherited Metab. Dis. 20*, 705−706.
9. Refsum, H., Ueland, P. M., Nygard, O., and Vollset, S. E. (1998) *Annu. Rev. Med. 49*, 31−62.
10. Mills, J. L., McPartlin, J. M., Kirke, P. N., Lee, Y. J., Conle, M. R., and Weir, D. G. (1995) *Lancet 345*, 149−151.
11. Miller, J. W. (1999) *Nutr. Rev. 57*, 126−129.
12. Seshadri, S., Beiser, A., Selhub, J., Jacques, P. F., Rosenberg, I. H., D'Agostino, R. B., Wilson, P. W., and Wolf, P. A. (2002) *N. Engl. J. Med. 346*, 476−483.
13. Clarke, R., Smith, A. D., Jobst, K. A., Refsum, H., Sutton, L., and Ueland, P. M. (1998) *Arch. Neurol. 55*, 1449−1455.
14. Jacques, P. F., Bostom, A. G., Williams, R. R., Ellison, R. C., Eckfeldt, J. H., Rosenberg, I. H., Selhub, J., and Rozen, R. (1996) *Circulation 93*, 7−9.
15. Wilson, A., Platt, R., Wu, Q., Leclerc, D., Christensen, B., Yang, H., Gravel, R. A., and Rozen, R. (1999) *Mol. Genet. Metab. 67*, 317−323.
16. Brown, C. A., McKinney, K. Q., Kaufman, J. S., Gravel, R. A., and Rozen, R. (2000) *J. Cardiovasc. Risk 7*, 197−200.
17. Hobbs, C. A., Sherman, S. L., Yi, P., Hopkins, S. E., Torfs, C. P., Hine, R. J., Pogribna, M., Rozen, R., and James, S. J. (2000) *Am. J. Hum. Genet. 67*, 623−630.
18. O'Leary, V. B., Parle-McDermott, A., Molloy, A. M., Kirke, P. N., Johnson, Z., Conley, M., Scott, J. M., and Mills, J. L. (2002) *Am. J. Med. Genet. 107*, 151−155.
19. Gaughan, D. J., Kluijtmans, L. A., Barbaux, S., McMaster, D., Young, I. S., Yarnell, J. W., Evans, A., and Whitehead, A. S. (2001) *Atherosclerosis 157*, 451−456.
20. Chen, Z., and Banerjee, R. (1998) *J. Biol. Chem. 273*, 26248−26255.
21. Chen, Z. (1998) Ph.D. Thesis, University of Nebraska, Lincoln, NE.
22. Klatt, P., Schmidt, K., Werner, E. R., and Mayer, B. (1996) *Methods Enzymol. 268*, 359−365.
23. Aasa, R., and Vanngard, T. (1975) *J. Magn. Reson. 19*, 308−315.
24. Rupp, H., Rao, K. K., Hall, D. O., and Cammack, R. (1978) *Biochim. Biophys. Acta 537*, 255−260.
25. Steyn-Parve, E. P., and Beinert, H. (1958) *J. Biol. Chem. 233*, 843−852.
26. Siegel, J. M., Montgomery, G. A., and Bock, R. M. (1959) *Arch. Biochem. Biophys. 82*, 288−299.
27. Palmer, G., Muller, F., and Massey, V. (1969) *Flavin and Flavoproteins*, University Park Press, Baltimore.
28. Ehrenberg, A., Muller, F., and Hemmerich, P. (1967) *Eur. J. Biochem. 2*, 286−293.
29. Muller, F., Hemmerich, P., Ehrenberg, A., Palmer, G., and Massey, V. (1970) *Eur. J. Biochem. 14*, 185−196.
30. Paine, M. J., Garner, A. P., Powell, D., Sibbald, J., Sales, M., Pratt, N., Smith, T., Tew, D. G., and Wolf, C. R. (2000) *J. Biol. Chem. 275*, 1471−1478.
31. Garner, A. P., Paine, M. J., Rodriguez-Crespo, I., Chinje, E. C., Ortiz De Montellano, P., Stratford, I. J., Tew, D. G., and Wolf, C. R. (1999) *Cancer Res. 59*, 1929−1934.
32. Vermilion, J. L., and Coon, M. J. (1978) *J. Biol. Chem. 253*, 8812−8819.
33. Rady, P. L., Szucs, S., Grady, J., Hudnall, S. D., Kellner, L. H., Nitowsky, H., Tyring, S. K., and Matalon, R. K. (2002) *Am. J. Med. Genet. 107*, 162−168.
34. Hall, D. A., Jordan-Starck, T. C., Loo, R. O., Ludwig, M. L., and Matthews, R. G. (2000) *Biochemistry 39*, 10711−10719.
35. Hall, D. A., Vander Kooi, C. W., Stasik, C. N., Stevens, S. Y., Zuiderweg, E. R., and Matthews, R. G. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 9521−9526.
36. Bandarian, V., Pattridge, K. A., Lennon, B. W., Huddler, D. P., Matthews, R. G., and Ludwig, M. L. (2002) *Nat. Struct. Biol. 9*, 53−56.
37. Hustad, S., Ueland, P. M., Vollset, S. E., Zhang, Y., Bjorke-Monsen, A. L., and Schneede, J. (2000) *Clin. Chem. 46*, 1065−1071.
38. Yamada, K., Chen, Z., Rozen, R., and Matthews, R. G. (2001) *Proc. Natl. Acad. Sci. U.S.A. 98*, 14853−14858.
39. Louerat-Oriou, B., Perret, A., and Pompon, D. (1998) *Eur. J. Biochem. 258*, 1040−1049.

40. Vermilion, J. L., and Coon, M. J. (1978) *J. Biol. Chem. 253*, 2694−2704.
41. Munro, A. W., Noble, M. A., Robledo, L., Daff, S. N., and Chapman, S. K. (2001) *Biochemistry 40*, 1956−1963.
42. Noble, M. A., Munro, A. W., Rivers, S. L., Robledo, L., Daff, S. N., Yellowlees, L. J., Shimizu, T., Sagami, I., Guillemette, J. G., and Chapman, S. K. (1999) *Biochemistry 38*, 16413−16418.
43. Sevrioukova, I., Shaffer, C., Ballou, D. P., and Peterson, J. A. (1996) *Biochemistry 35*, 7058−7068.
44. Stuehr, D., Pou, S., and Rosen, G. M. (2001) *J. Biol. Chem. 276*, 14533−14536.
45. Hoover, D. M., Jarrett, J. T., Sands, R. H., Dunham, W. R., Ludwig, M. L., and Matthews, R. G. (1997) *Biochemistry 36*, 127−138.
46. Chen, Z., Crippen, K., Gulati, S., and Banerjee, R. (1994) *J. Biol. Chem. 269*, 27193−27197.
47. Drummond, J., Huang, S., Blumenthal, R. M., and Matthews, R. G. (1993) *Biochemistry 32*, 9290−9295.
48. Fujii, K., Galivan, J. H., and Huennekens, F. M. (1977) *Arch. Biochem. Biophys. 178*, 662−670.
49. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis 18*, 2714−2723.

BI020536S