Unique Holoenzyme Dimers of the Tetrameric Enzyme *Escherichia coli* Methylenetetrahydrofolate Reductase: Characterization of Structural Features Associated with Modulation of the Enzyme's Function†

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ABSTRACT: Impaired functioning of methylenetetrahydrofolate reductase (MTHFR) can cause high levels of homocysteine in plasma or hyperhomocysteinemia, which is an independent risk factor for cardiovascular diseases and neural tube defects. We have studied in detail the effect of modulation of hydrophobic and electrostatic interactions of *Escherichia coli* MTHFR on its structure and function. Alterations in hydrophobic interactions of MTHFR, using urea, lead to dissociation of the native tetramer, resulting in stabilization of enzymatically active holoenzyme dimers followed by unfolding of the holoenzyme dimer to the denatured monomer along with dissociation of FAD from the enzyme. This is the first report of an enzymatically active dimer of *E. coli* MTHFR and suggests that the dimer rather than tetramer is the smallest functionally active unit of the enzyme. Furthermore, these results also demonstrate that dissociation of the FAD cofactor from the enzyme occurs only on unfolding of the dimer to denatured monomers. Modulation of electrostatic interactions, using NaCl, leads to dissociation of the native enzyme, resulting in stabilization of an enzymatically inactive partially unfolded holoenzyme dimer. Comparative analysis of loss of enzymatic activity and changes in structural features of MTHFR demonstrate a very good correlation between enhanced flexibility of the enzyme-bound FAD and loss of enzymatic activity, suggesting the importance of rigidity of the FAD cofactor in maintenance of the enzymatic activity of MTHFR.

Homocysteine is an emerging risk factor for cardiovascular diseases and neural tube defects in humans (*1*, *2*), as elevated plasma homocysteine concentrations are associated with these diseases. Folate treatment decreases homocysteine levels and dramatically reduces the incidence of neural tube defects (*3*, *4*). The flavoprotein methylenetetrahydrofolate reductase (MTHFR)[1] is a likely target for the actions of folate as it catalyzes the reduction of 5,10-methylenetetrahydrofolate (CH_2-H_4folate) to 5-methyltetrahydrofolate (CH_3-H_4folate) using NAD(P)H (*5*). MTHFR is the only route for synthesis of the CH_3-H_4folate that is the predominant circulating form of folate and the main carbon donor for the remethylation of homocysteine to methionine. Impaired functioning of MTHFR due to mutations in this gene has been correlated with elevated plasma levels of homocysteine or hyperhomocysteinemia in humans (*6*, *7*).

The MTHFR proteins and genes of *Escherichia coli* and mammals including humans have been characterized (*8*−*12*). The mammalian MTHFRs are homodimers of 77 kDa subunits that employ NADPH as a reducing agent (*9*). The polypeptide chain of mammalian MTHFR contains an N-terminal catalytic domain and a C-terminal regulatory domain (*13*). Determinants of binding for FAD, NADPH, and CH_2-H_4folate are located in the N-terminal portion of the protein. The subunits of MTHFRs of enteric bacteria are shorter chains of about 300 residues in length. The MTHFRs of *E. coli* are homotetramers of 33 kDa subunits (273 residues in length) that prefer NADH to NADPH (*12*). The primary sequence of the subunit of *E. coli* MTHFR can be aligned with the N-terminal catalytic domain of the human MTHFR and shows about 30% identity (*11*). This level of primary sequence conservation predicts that the catalytic domains of human and *E. coli* enzymes will possess similar structure (*8*).

The structure of *E. coli* MTHFR, the first example of an $\beta_8\alpha_8$ barrel that binds FAD, has been reported (*8*). The enzyme is a tetramer of identical subunits. The X-ray structure reveals an unusual structure for the *E. coli* MTHFR tetramer with four subunits arranged in a planar rosette and only 2-fold rotational symmetry (*8*). The cofactor FAD is bound at the C-termini of the β strands. The riboflavin moiety lies within the barrel with its plane parallel to staves of the barrel and its *si* face exposed to the solvent. As flavin is essential for transferring electrons between NADH and CH_2-H_4folate, flavin dissociation is accompanied by the loss of enzymatic activity (*8*, *10*). The *E. coli* MTHFR shows an interesting property in that the native tetramer of the enzyme dissociates into dimers preceding flavin release at low enzyme concentration (*8*, *10*).

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[1] Abbreviations: MTHFR, methylenetetrahydrofolate reductase; FAD, flavin adenine dinucleotide; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; CD, circular dichroism.

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We have carried out extensive studies on the effect of modulation of hydrophobic and electrostatic interactions on functional and structural (quaternary and secondary) properties of *E. coli* MTHFR. Comparative analysis of changes in various structural parameters and loss of enzymatic activity of MTHFR on treatment with increasing concentrations of urea or NaCl has been carried out. Furthermore, the structural details of the urea-induced unfolding of MTHFR have also been discussed.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, and were of the highest purity available.

Methods

Overexpression and Purification of Methylenetetrahydrofolate Reductase. The MTHFR was overexpressed in the BL21(DE3)recD$^+$ (Novagen) strain of *E. coli* cells. The overproduced protein was purified to homogeneity with slight variations in the previously described procedure (*12*). The pH of all the buffers was 8.0 instead of 7.6. The purity of the purified enzyme was evaluated on SDS−PAGE (*14*) followed by Coomassie blue staining and was found to be about 99% pure. Both native and C-terminally hexahistidine-tagged MTHFRs were cloned, overexpressed, and purified. The basic structural properties and changes associated with the denaturation of enzyme with urea and NaCl were studied using both enzymes and were found to be similar. Hence, the C-terminally hexahistidine-tagged enzyme was used for the detailed study, as its purification was easier.

Urea and NaCl Treatment of MTHFR. MTHFR in 50 mM phosphate buffer, pH 7.2, containing 0.3 mM EDTA and 10% glycerol, was incubated in the absence and presence of increasing concentrations of urea or sodium chloride for 6 h at 25 °C before the measurements were made.

Enzymatic Activity. Methylenetetrahydrofolate reductase activity was determined by the NADH−menadione oxidoreductase assay method as described previously (*15*). For studies using increasing concentrations of urea or NaCl, the assay buffer contained concentrations of urea or NaCl similar to those in which the enzyme was incubated.

Fluorescence Spectroscopy. Fluorescence spectra were recorded with a Perkin-Elmer LS 50B spectrofluorometer in a 5 mm path length quartz cell. MTHFR dissolved in 50 mM phosphate buffer, in the absence and presence of increasing concentrations of NaCl or urea, was incubated for 6 h at 25 °C before the spectra were recorded. The protein concentration was 3.0 μM for all experiments, and the measurements were carried out at 25 °C. For monitoring FAD fluorescence, the excitation wavelength was 380 nm, and the spectra were recorded between 450 and 650 nm. The polarization was measured at a fixed wavelength of 525 nm.

Circular Dichroism Measurements. CD measurements were made with a JASCO 810 spectropolarimeter calibrated with ammonium (+)-10-camphorsulfonate. The results are expressed as the mean residual ellipticity [θ], which is defined as $[\theta] = 100\theta_{obs}/(lc)$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in moles of residue per liter, and l is the path length in centimeters. The CD spectra were measured at an enzyme concentration of 3.0 μM with a 1 mm cell at 25 °C. The values obtained were normalized by subtracting the baseline recorded for buffer having the same concentration of denaturant under similar conditions.

Cross-Linking Using Glutaraldehyde. MTHFR (2 μM) was incubated in the desired concentrations of urea or NaCl for 6 h. Glutaraldehyde was added to a final concentration of 1%. After 5 min of incubation at 25 °C, the reaction was quenched by the addition of 22 mg of glycine (3 mL reaction mix). After incubation for 20 min at 25 °C, 2 μL of 10% deoxycholic acid was added. The protein was precipitated by addition of orthophosphoric acid so as to bring the pH to 2−2.5. The sample was centrifuged, and the pellet was suspended in sample buffer for analysis on 8% SDS−PAGE.

Size Exclusion Chromatography. Gel filtration experiments were carried out on a Superdex 200HR 10/30 column (manufacturer's exclusion limit 600 kDa for proteins) on AKTA FPLC (Amersham Pharmacia Biotech, Sweden). The MTHFR solution (3 μM) was incubated at the desired NaCl or urea concentration for 6 h at 25 °C. Then 200 μL of this sample was loaded on the column and run using the same buffer at a flow rate of 0.4 mL/min with detection at 280 nm.

Refolding of MTHFR. MTHFR was incubated in various fixed concentrations of urea or NaCl for 6 h at 25 °C. The enzyme was refolded by the dilution method (10 times dilution at 4 °C) in 50 mM phosphate buffer, pH 7.2. The refolding efficiency was measured by monitoring the enzymatic activity after refolding and presented as percent activity, taking the enzymatic activity of the native enzyme as 100%.

RESULTS

We have studied the effect of urea- and sodium chloride-induced changes on the structural and functional properties of MTHFR.

Time-dependent changes in structural parameters and enzymatic activity of MTHFR at 0.5, 2.5, and 5 M urea and 0.5 and 1.6 M NaCl concentrations were monitored to standardize the incubation time required for achieving equilibrium under these conditions. Under all of the conditions studied, the changes occurred within a maximum of 5 h with no further alteration up to 12 h (data not shown). These observations demonstrate that an incubation time of about 5 h is sufficient for achieving equilibrium under any condition studied.

Urea-Induced Changes on Molecular Properties of MTHFR. Enzyme activity can be regarded as the most sensitive probe to study the changes in the enzyme conformation during various treatments as it reflects subtle readjustments at the active site, allowing very small conformational variations of an enzyme structure to be detected. Figure 1A shows the effect of increasing concentrations of urea on the enzymatic activity of MTHFR in the urea concentration range of 0−3.5 M. A biphasic curve corresponding to two independent transitions centered at about 1.0 and 2 M urea, respectively, was observed. About 60% loss of enzymatic activity was found to be associated with the first transition, and a complete loss of activity was observed above 2.5 M urea. These observations suggest that an intermediate of MTHFR is stabilized at low concentrations of urea.

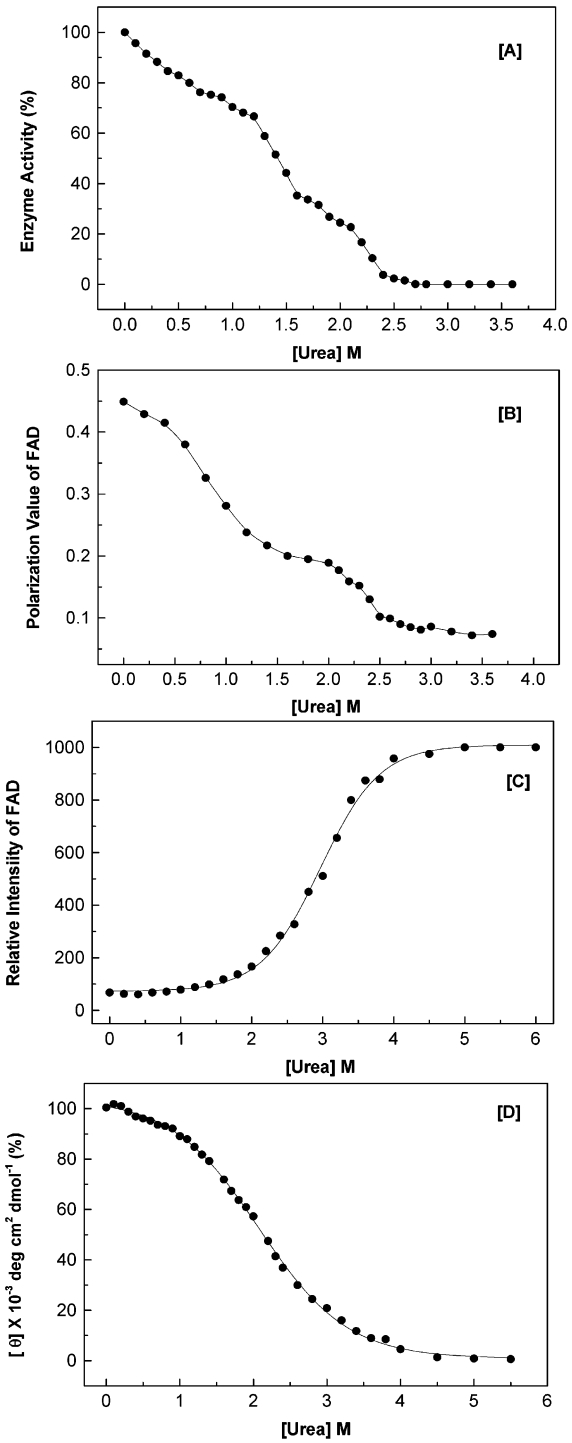

FIGURE 1: Changes in functional and structural properties of *E. coli* MTHFR in the presence of increasing concentration of urea at pH 7.2 and 25 °C. (A) Changes in enzymatic activity of MTHFR on treatment with increasing concentration of urea. The data in the figure are expressed in terms of relative activity using the activity of native enzyme as reference (100%). (B) Changes in FAD fluorescence polarization of MTHFR on treatment with increasing urea concentration as monitored by the fluorescence emission at 525 nm and excitation at 380 nm. (C) Changes in FAD fluorescence of MTHFR on treatment with increasing urea concentration as monitored by fluorescence emission at 525 nm; the excitation wavelength was 380 nm. (D) Urea-induced changes in the secondary structure of MTHFR as monitored by following changes in ellipticity at 222 nm obtained from the far-UV CD curves of MTHFR at increasing concentrations of urea. The data are represented as percentage of ellipticity at 222 nm, taking the value observed for native MTHFR as 100%.

Optical spectroscopic studies on MTHFR in the presence of increasing urea concentrations were performed to study the effect of denaturant on the structural properties of the enzyme.

Studies on various flavoproteins have reported that the fluorescent prosthetic group FAD or FMN exhibits different spectral characteristics in different proteins, reflecting the specific environmental property of isoalloxazine, which is the chromophore present in the molecule (*16*). For this reason the FAD group has been used as a natural marker to probe the dynamical microenvironment of the flavin fluorophore in flavoproteins (*17*, *18*). MTHFR contains four tightly bound, but noncovalently linked, flavin adenine dinucleotide (FAD) molecules (*8*). In the native conformation of MTHFR the FAD moiety is buried; hence, a significantly high polarization and a weak fluorescence intensity of FAD are observed for emission at 525 nm.

Figure 1B summarizes the changes in FAD fluorescence polarization of MTHFR with increasing urea concentration. A biphasic dependence of FAD fluorescence polarization with increasing urea concentrations (0−3.5 M) was observed, indicating the presence of two independent transitions centered at 0.75 and about 2.2 M urea, respectively, under these conditions. These results substantiate the observation of stabilization of an intermediate of MTHFR at low urea concentrations, as indicated from enzymatic activity measurements reported above. A loss of about 55% in fluorescence polarization of FAD was associated with the first transition. This decrease in polarization is as a result of increased local freedom of rotation of enzyme-bound FAD due to loss of rigidity at the FAD binding site in the enzyme molecule under these conditions. At urea concentrations of 3 M and above, a complete loss of polarization was observed, suggesting that under these conditions the enzyme molecule is denatured and perhaps the FAD gets dissociated from the enzyme molecule.

Figure 1C summarizes the changes in FAD fluorescence intensity of MTHFR with increasing urea concentration. A sigmoidal dependence of FAD fluorescence intensity with increasing urea concentration was observed. A slight linear enhancement in FAD fluorescence of the native enzyme was observed between 0 and 1.2 M urea. However, between 2 and 4.5 M urea, an enhancement of about 10 times in FAD fluorescence intensity was observed. Enhancement of FAD fluorescence intensity of MTHFR has been associated with release of the FAD molecule from the enzyme (*8*, *10*); hence, the above observations suggest that treatment of MTHFR with higher concentrations of urea induces dissociation of FAD from the enzyme.

The FAD fluorescence studies are very interesting. A significant loss (about 55%) of FAD polarization is observed between 0 and 1 M urea, but a significantly large enhancement in the FAD fluorescence intensity of native MTHFR is not observed under these conditions. This suggests that although a significant enhancement in flexibility of enzyme-bound FAD occurs on treatment of MTHFR with low urea concentrations, there is no significant enhancement in the exposure of the buried FAD molecule in the native enzyme toward the solvent under these conditions. However, at higher urea concentrations a significantly large enhancement in FAD fluorescence intensity as well as complete loss of fluorescence polarization is observed, suggesting that under these

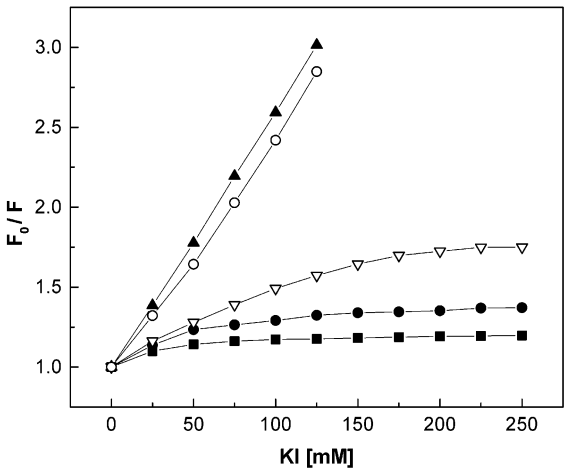

FIGURE 2: Stern−Volmer plot of FAD fluorescence quenching by KI of native MTHFR (filled squares), 1 M urea-stabilized enzyme (filled circles), 4 M urea-stabilized enzyme (open circles), 1.2 M NaCl-stabilized enzyme (open inverted triangles), and free FAD (filled triangles). Quenching with KI was performed at pH 7.2 and 25 °C by addition of 3 μL of KI (4 M stock solution) to 700 μL of sample, to obtain a final KI concentration between 0 and 250 mM, and by monitoring the decrease in fluorescence emission at 525 nm. An enzyme concentration of 3 μM was used in the study.

conditions the FAD is probably dissociated from the enzyme, resulting in exposure of FAD molecules to solvent. These observations are further supported by the studies on FAD fluorescence quenching using KI. Figure 2 shows the fractional fluorescence of FAD in native and 1 and 4 M urea-stabilized MTHFR as a function of KI concentration. For both native and 1 M urea-stabilized MTHFR, a small quenching of FAD by KI was observed, suggesting that under these conditions the FAD molecule is buried in the protein interior and is only partially accessible to the quencher (*19*). However, for 4 M urea-stabilized MTHFR, fluorescence quenching similar to that for free FAD is observed, thus suggesting that in 4 M urea-treated MTHFR the FAD molecule is dissociated from the enzyme and is freely available in the solvent. The results of FAD fluorescence studies in conjunction with studies of enzymatic activity (Figure 1A) demonstrate that for MTHFR loss of enzymatic activity is closely related with loss of rigidity of the enzyme-bound FAD and not with dissociation of FAD from the enzyme.

Far-UV CD studies on urea-induced changes in MTHFR were carried out to study the effect of urea on the secondary structure of the enzyme. In the far-UV region, the CD spectrum of native MTHFR shows the presence of substantial α-helical conformation (*19*). Figure 1D summarizes the effect of increasing urea concentration on the ellipticity of MTHFR at 222 nm for native MTHFR. Up to a urea concentration of about 1.0 M, a slight decrease (about 10%) in ellipticity at 222 nm of native MTHFR was observed. However, between urea concentrations of 1.2 and 4 M, a large gradual decrease in ellipticity at 222 nm was observed, suggesting that the transition corresponds to the urea-induced major unfolding of MTHFR. The significant loss of secondary structure of MTHFR induced by urea corresponds to the second transition observed at high urea concentration for enzymatic activity and FAD polarization studies.

The urea-induced changes in the molecular properties of MTHFR as reported above demonstrate that at low concentrations of urea (≤1 M) a form of the enzyme having an altered active site and FAD microenvironment and slightly less secondary structure than the native tetrameric enzyme is stabilized. The major part of the urea-induced transition observed for MTHFR as monitored by loss of secondary structure (Figure 1D) and changes in FAD fluorescence intensity (Figure 1C) corresponds to the second transition (at higher urea concentration) observed for changes in enzymatic activity and FAD polarization under similar conditions. This suggests that higher concentrations of urea bring about major unfolding of MTHFR. Furthermore, the enhancement in FAD fluorescence intensity, indicating dissociation of FAD from the enzyme molecule, correlated well with loss of secondary structure as monitored by CD ellipticity at 222 nm. This suggests that the FAD molecule undergoes dissociation from the enzyme molecule only on major unfolding of the enzyme.

Sodium Chloride-Induced Changes on Molecular Properties of MTHFR. Figure 3 shows sodium chloride-induced changes in functional and structural features of MTHFR as studied by measurements of the enzymatic activity and optical spectroscopy on treatment of MTHFR with increasing concentrations of NaCl (0−1.8 M). The effect of NaCl treatment on the enzymatic activity of MTHFR is summarized in Figure 3A. A slight enhancement in enzymatic activity (maximum of about 10%) of MTHFR was observed on treatment of enzyme with low concentrations (up to 0.2 M) of NaCl. However, between 0.2 and 1 M NaCl a sharp decrease in enzymatic activity was observed. Panels B and C of Figure 3 summarize the effect of increasing concentrations of NaCl on the FAD microenvironment as monitored by changes in FAD polarization and fluorescence intensity, respectively. An exponential decrease in polarization values from 0.405 to 0.30 was observed between 0 and 1.2 M NaCl (Figure 3B). This change in fluorescence polarization is similar to that observed for the first transition during urea denaturation of MTHFR (Figure 1B). Under similar conditions, an almost linear enhancement in the FAD fluorescence intensity with increasing NaCl concentration was observed (Figure 3C). However, the maximum enhancement in FAD fluorescence intensity associated with this transition was significantly less (only about 2.6 times) compared to that observed (10 times; Figure 1C) for the transition of native enzyme to unfolded enzyme. These observations indicate that NaCl treatment of MTHFR leads to enhanced exposure of enzyme-bound FAD toward solvent molecules. This conclusion is also supported by the studies on FAD fluorescence quenching using KI (Figure 2), where a significantly enhanced quenching of FAD fluorescence for 1.2 M NaCl-treated MTHFR as compared to the native enzyme was observed. The enhanced solvent exposure of FAD for NaCl-treated MTHFR is probably as a result of NaCl-induced partial unfolding of the enzyme. The partial unfolding of MTHFR by NaCl is further confirmed by the results from the CD studies where changes in CD ellipticity at 222 nm were monitored at increasing NaCl concentration (Figure 3D). A sigmoidal dependence of loss of secondary structure with increasing NaCl concentration was observed between 0.6 and 1.6 M NaCl. However, only about 25% loss of secondary structure was found to be associated with the NaCl-induced transition of MTHFR, thus confirming that NaCl induces only partial unfolding of MTHFR.

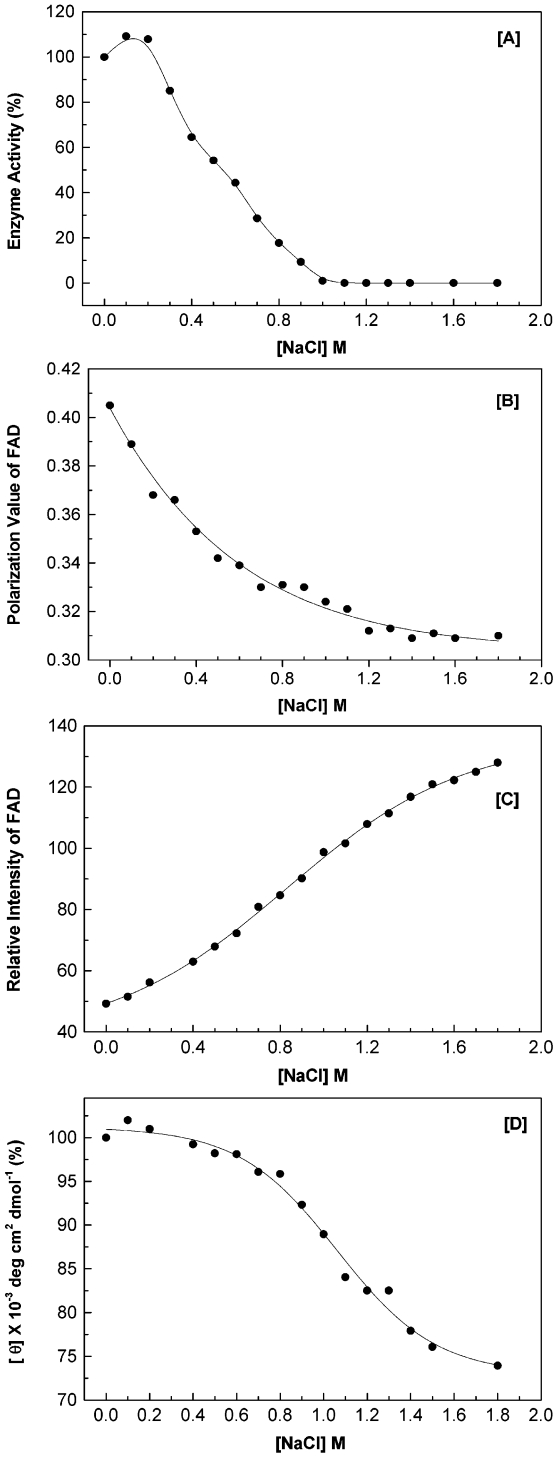

FIGURE 3: Changes in functional and structural properties of *E. coli* MTHFR in the presence of increasing concentration of sodium chloride at pH 7.2 and 25 °C. (A) Changes in enzymatic activity of MTHFR on treatment with increasing concentrations of NaCl. The data in the figure are expressed in terms of relative activity using the activity of native enzyme as reference (100%). (B) Changes in FAD fluorescence polarization of MTHFR on treatment with increasing NaCl concentrations as monitored by fluorescence emission at 525 nm; the excitation wavelength was 380 nm. (C) Changes in FAD fluorescence intensity of MTHFR on treatment with increasing NaCl concentrations as monitored by fluorescence emission at 525 nm; the excitation wavelength was 380 nm. (D) NaCl-induced changes in secondary structure of MTHFR as monitored by following the changes in ellipticity at 222 nm obtained from the far-UV CD curves of MTHFR at increasing concentrations of urea. The data are represented as percentage of ellipticity at 222 nm, taking the value observed for native MTHFR as 100%.

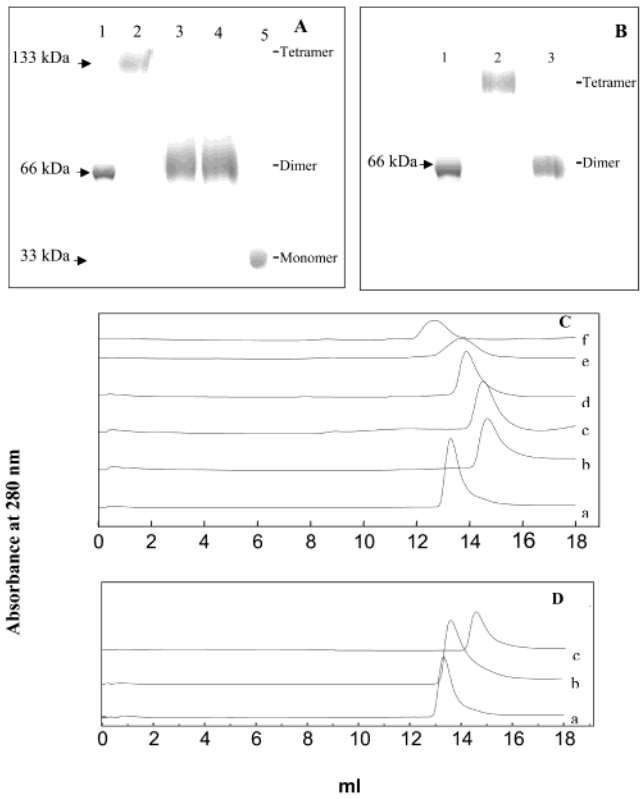

FIGURE 4: Effect of urea and NaCl on the subunit configuration and molecular dimension of MTHFR. The SDS−PAGE profiles of glutaraldehyde cross-linked samples of MTHFR and on incubation with increasing concentration of urea (A) and 1.0 M NaCl (B). In panel A, lanes 1−5 are the 66 kDa marker, glutaraldehyde cross-linked native MTHFR, and 1, 2, and 6 M urea-treated MTHFR, respectively. In panel B lanes 1−3 are the 66 kDa marker, glutaraldehyde cross-linked native MTHFR, and 1.0 M NaCl-treated MTHFR, respectively. Size exclusion chromatographic profile of the Superdex 200HR column for (C) urea-treated MTHFR and (D) NaCl-treated MTHFR. In panel C, curves a−f represent samples of native and 1.0, 2.0, 3.5, 5.0, and 6.0 M urea-treated MTHFR, respectively. In panel D, curves a−c represent samples of native and 0.65 M and 1.2 M NaCl-treated MTHFR, respectively. The experimental details are given in Experimental Procedures.

The results presented above demonstrate that NaCl treatment of MTHFR leads to enhanced exposure of the enzyme-bound FAD toward the solvent along with loss of about one-fourth of the secondary structure of the enzyme. These observations collectively suggest that alteration of electrostatic interactions in MTHFR by NaCl results in partial unfolding of native enzyme.

Urea- or NaCl-Induced Changes in Quaternary Structure and Molecular Dimensions of MTHFR. (A) Cross-Linking Studies. Glutaraldehyde cross-linking studies were carried to study the effect of urea and NaCl treatment on the subunit configuration of MTHFR and are summarized in Figure 4A,B. For MTHFR treatment with a urea concentration of 1 and 2 M only dimers were observed (Figure 4A), suggesting that under these conditions urea induces dissociation of the native tetramer of MTHFR, resulting in stabilization of dimers. At a high urea concentration of about 6 M, only monomers of MTHFR were observed (Figure 4A).

Figure 4B summarizes the effect of NaCl on the subunit composition of MTHFR. Glutaraldehyde cross-linking of 1.2 M NaCl-treated MTHFR showed the presence of only dimers, suggesting that NaCl treatment results in dissociation of the native tetramer of MTHFR into dimers.

(B) Size Exclusion Chromatography. The effect of urea and NaCl on the molecular dimensions of native MTHFR at increasing concentrations of urea and NaCl was monitored by size exclusion chromatography on a Superdex S-200 column. Figure 4C summarizes the results of gel permeation experiments on MTHFR in the presence and absence of increasing concentrations of urea at 25 °C on a Superdex 200HR 10/30 column (Amersham Pharmecia Biotech, Sweden; manufacturer's exclusion limit 600 kDa for proteins). For the native MTHFR tetramer, a single peak centered at 13.5 mL was observed, which is slightly greater than the retention volume of 13.0 mL observed in this column for a molecular mass marker of 160 kDa (Amersham Pharmacia Biotech, Sweden) under similar conditions. As the molecular mass of native MTHFR is 133 kDa, these observations indicate that native MTHFR under the conditions studied is a tetramer, which is in agreement with the results obtained from glutaraldehyde cross-linking (Figure 4A) and literature reports (*8*). However, for 1 M urea-treated MTHFR, a single peak with a significantly higher retention volume of 14.6 mL was observed, indicating a large decrease in molecular dimensions of the enzyme under these conditions. For the molecular mass marker of 66 kDa (Amersham Pharmacia Biotech, Sweden), a retention volume of 14.6 mL was observed. As the dimer of MTHFR has a molecular mass of about 66 kDa, these observations suggest that the reduced hydrodynamic radius observed for 1 M urea-stabilized MTHFR is due to dissociation of the native enzyme tetramer to a dimer under these conditions. These conclusions are also supported by the results from glutaraldehyde cross-linking studies (Figure 4A). With increasing urea concentration to 2 M, a slight decrease in the retention volume of MTHFR was observed, indicating a partial unfolding of MTHFR by urea under these conditions. However, at 2 M urea dimeric species of MTHFR were observed by glutaraldehyde cross-linking (Figure 4A). These observations collectively suggest that at 2 M urea a partially unfolded dimer of MTHFR is stabilized. At 6 M urea, a single peak with a retention volume of 12.6 mL was observed, which is slightly less than the retention volume of 13.5 mL observed for the native tetramer. These observations indicate that the enzyme under these conditions has a slightly enhanced molecular dimension as compared to the native tetrameric enzyme. However, at 6 M urea only monomeric species of MTHFR were found to be stabilized (Figure 4A). Monomeric species of MTHFR having molecular dimensions greater than the native tetramer can occur only when the unfolded monomers of MTHFR are present. The presence of unfolded enzyme at 6 M urea is also supported by the results of structural studies presented above (Figure 1).

Figure 4D summarizes the results of gel permeation experiments on MTHFR in the presence and absence of increasing concentrations of NaCl at 25 °C on a Superdex 200HR 10/30 column. For NaCl treatment of MTHFR with increasing concentrations of NaCl up to 0.75 M, no significant change in retention volume of the native tetramer of MTHFR was observed, indicating no significant alteration in the molecular dimension of native MTHFR under these conditions. However, for treatment of MTHFR with 1.0 M NaCl, a single peak with a significantly higher retention volume of 14.5 mL was observed, indicating the stabilization of a dimeric species under these conditions, as discussed above and also observed in the glutaraldehyde cross-linking experiment (Figure 4B).

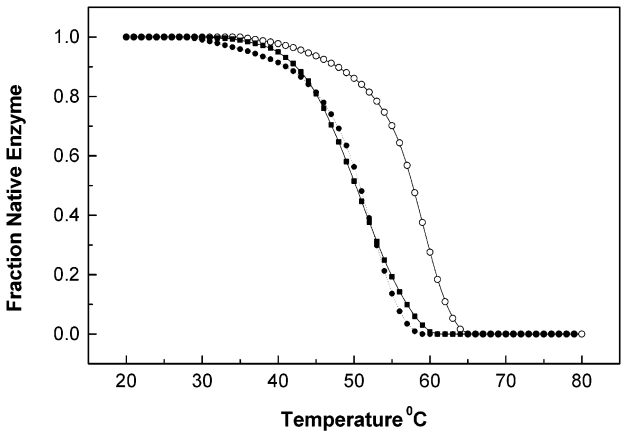

FIGURE 5: Thermal denaturation profiles of the native (open circles), 1 M urea-stabilized holoenzyme dimer (filled circles), and 1 M NaCl-stabilized holoenzyme dimer (filled squares) of MTHFR as monitored by changes in CD ellipticity at 222 nm with increasing temperature between 5 and 100 °C. An enzyme concentration of 10 μM was used for the study. For all of the curves, a linear extrapolation of the baselines in the pre- and posttransitional regions was used to determine the fraction of unfolded protein within the transition region by assuming a two-state mechanism of unfolding.

Table 1: Properties of Urea- and NaCl-Stabilized Holoenzyme Dimers Compared to the Native Tetramer of *E. coli* MTHFR

properties	urea-stabilized dimer	NaCl-stabilized dimer
enzymatic activity	70% activity	inactive
FAD polarization	60% loss	25% loss
secondary structure	10% loss	25% loss
FAD fluorescence intensity	slightly enhanced	2.6 times enhanced
refolding efficiency	100%	0%
thermal stability	~7 °C lower	~7 °C lower

Thermal Denaturation Profiles of Urea- and NaCl-Stabilized Dimers of MTHFR. Studies on the thermal denaturation of dimers of MTHFR stabilized at 1 M urea and 1.2 M NaCl were carried out. Figure 5 represents the change in ellipticity at 222 nm for 1 M urea-stabilized and 1.2 M NaCl-stabilized dimers of MTHFR at increasing temperature. For native MTHFR at pH 7.2, a T_m of about 58 °C was observed, which is in accordance with the earlier report (*8*). However, for the 1 M urea-stabilized or 1.2 M NaCl-stabilized dimer of MTHFR, a T_m of 51 °C was observed. These observations demonstrate that the dimeric species of MTHFR are thermally less stable than the native tetramer of MTHFR.

Refolding Studies. The refolding efficiency of the various holoenzyme dimers of MTHFR stabilized under different conditions was also studied. The 1 M urea-stabilized enzymatically active dimer showed 100% refolding (Table 1) as observed by recovery of enzymatic activity on removal of urea. However, the holoenzyme dimers stabilized by modulation of electrostatic interactions (the NaCl-stabilized dimers) were found to undergo irreversible modification as no recovery of activity was observed on removal of NaCl.

DISCUSSION

The X-ray structure of *E. coli* MTHFR reveals an unusual structure for the native tetramer of the enzyme, with four

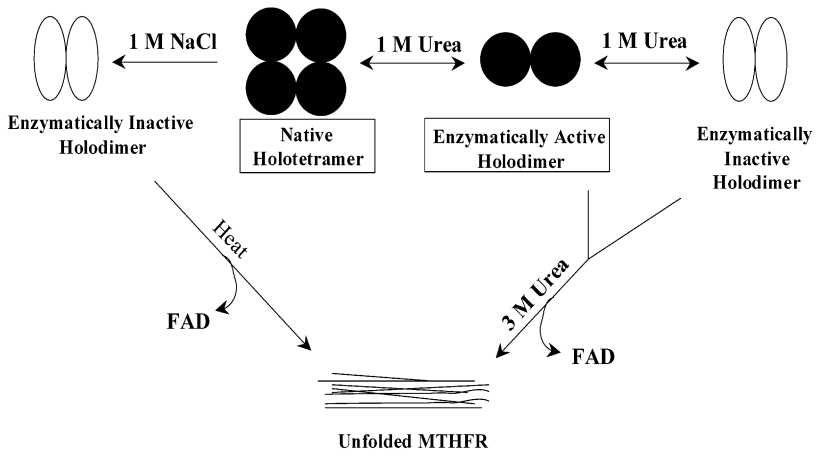

FIGURE 6: Schematic diagram summarizing the urea- and NaCl-induced changes in the MTHFR subunit structure and FAD release from the enzyme.

subunits arranged in a plane rosette and only 2-fold rotational asymmetry (*8*). Each subunit of MTHFR interacts extensively with one of its neighbors and much less with the other, favoring dissociation of the tetramer into dimers rather than monomers (*8*). The experimental support for this suggestion comes from the studies presented in this paper (Figure 6) where modulation of either hydrophobic interactions, using urea (*20*), or electrostatic interactions, using NaCl, of MTHFR was found to result in stabilization of holoenzyme dimers due to dissociation of the native tetrameric enzyme under these conditions.

An unexpected and novel finding of our work is the first time report of an enzymatically active holodimer of MTHFR which was stabilized on treatment of MTHFR with 1 M urea (Figure 6). The urea-stabilized holodimer showed about 70% enzymatic activity compared to the native enzyme. Only a slight loss of secondary structure was observed on dissociation of the tetramer to dimer, as the urea-stabilized holoenzyme dimer showed secondary structure slightly lesser than that of the native tetramer (Table 1). The urea-stabilized holoenzyme dimer showed complete reassociation into a native tetramer on removal of urea. However, the enzymatically active holodimer of MTHFR showed slightly decreased thermal stability as compared to the native tetramer (Figure 5). Besides the enzymatically active holodimer, an enzymatically inactive holodimer of MTHFR was also observed. This was stabilized on modulation of electrostatic interactions of MTHFR by treatment of the enzyme with about 1.2 M NaCl (summarized in Figure 6). Structural studies on the 1.2 M NaCl-stabilized holoenzyme dimers of MTHFR showed that a loss of about 25% of secondary structure of the native tetramer, along with movement of the buried FAD molecule toward the solvent, is associated with NaCl-induced dissociation of MTHFR. These observations suggest that the holoenzyme dimer of MTHFR that is stabilized on modulation of electrostatic interactions in the enzyme is probably a partially unfolded holodimer.

Comparative analysis of the changes in structural properties and loss of enzymatic activity of MTHFR on urea and NaCl treatment of MTHFR, as reported in this paper, demonstrates a very good correlation between the loss of enzymatic activity of MTHFR and loss of rigidity of the FAD molecule, suggesting a close relationship between these two properties. In MTHFR the cofactor FAD is bound at the C-termini of the β-strands present in the enzyme molecule (*8*). The riboflavin moiety of FAD lies within the β/α barrel with its plane parallel to the staves of the barrel and the *si* face exposed to the solvent (*21*). Investigation of the stereochemistry and mechanism of the NADPH$-CH_2$-H_4-folate oxidoreductase reaction with porcine MTHFR (*21*), which has catalytic properties similar to those of the *E. coli* enzyme (*22*), has demonstrated that in the reductive half-reaction NADPH binds at the *si* face of the FAD and the 4*S*-hydrogen of NADPH is transferred as hydride to N5 of the FAD cofactor (*21*). $NADP^+$ then dissociates from the enzyme. In the oxidative half-reaction, CH_2-H_4folate also binds at the *si* face of the reduced FAD, consistent with the ping-pong bi-bi mechanism (*22*). Due to the binding of the reactants to the *si* face of FAD, as described above, the orientation/stereochemistry of the FAD molecule in the active site would play an important role in maintenance of enzymatic activity of MTHFR. In the native enzyme the FAD molecule is buried and is in a rigid conformation (Figure 1A). Modulation of the hydrophobic or electrostatic interactions in the enzyme by urea or NaCl was found to lead to loss of rigidity at the FAD binding site, resulting in enhanced local freedom of rotation of the FAD cofactor. This would result in a modification in the orientation of the FAD molecule, which would subsequently lead to either a decrease in binding affinity or complete loss of binding of NADH or CH_2-H_4folate to FAD as a consequence of which either partial or complete loss of enzymatic activity will be observed.

Matthews and co-workers (*8*, *10*) have proposed a model depicting the steps involved in dissociation of MTHFR subunits and the FAD from the enzyme. According to this model, the dissociation of the tetramer into dimers precedes the FAD release from the enzyme (*10*). The urea-induced denaturation studies presented in this paper provide experimental support for the proposed model as they demonstrate that dissociation of the native tetramer of MTHFR leads to formation of the holoenzyme dimer. According to the results presented in this paper, the FAD dissociation from the enzyme occurs only on unfolding of the holodimer to monomer. This suggests that FAD is very tightly bound to the enzyme and is released only on extensive unfolding of enzyme. This suggestion is also supported by the structural analysis of the FAD binding site of the enzyme obtained

from the X-ray crystal structure of MTHFR. An intriguing feature of the FAD binding site in the MTHFR is the belt of side chains arching over the ribityl and phosphate groups and secured by the Arg118−Glu158 interaction that acts as a buckle. The β5−α5 connector, which includes Glu158 from the belt buckle, encloses the ribityl−diphosphate−ribose segments of the FAD. Hence, for release of FAD from the enzyme molecule, significant rearrangement or significant unfolding of the enzyme molecule must occur, which was indeed deduced from the structural studies presented in this paper.

The results presented in this paper demonstrate that modulation of either the hydrophobic or electrostatic interactions in *E. coli* MTHFR leads to dissociation of the native tetramer, resulting in stabilization of holodimers. FAD release from MTHFR has been conclusively demonstrated to occur only on unfolding of the holodimer to monomers.

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