Cation Binding and Thermostability of FTHFS Monovalent Cation Binding Sites and Thermostability of N^{10}-Formyltetrahydrofolate Synthetase from *Moorella thermoacetica*[†,‡]

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ABSTRACT: Formyltetrahydrofolate synthetase (FTHFS) from the thermophilic homoacetogen, *Moorella thermoacetica*, has an optimum temperature for activity of 55−60 °C and requires monovalent cations for both optimal activity and stabilization of tetrameric structure at higher temperatures. The crystal structures of complexes of FTHFS with cesium and potassium ions were examined and monovalent cation binding positions identified. Unexpectedly, NH_4^+ and K^+, both of which are strongly activating ions, bind at a different site than a moderately activating ion, Cs^+, does. Neither binding site is located in the active site. The sites are 7 Å apart, but in each of them, the side chain of Glu 98, which is conserved in all known bacterial FTHFS sequences, participates in metal ion binding. Other ligands in the Cs^+ binding site are four oxygen atoms of main chain carbonyls and water molecules. The K^+ and NH_4^+ binding site includes the carboxylate of Asp132 in addition to Glu98. Mutant FTHFS's (E98Q, E98D, and E98S) were obtained and analyzed using differential scanning calorimetry to examine the effect of these mutations on the thermostability of the enzyme with and without added K^+ ions. The addition of 0.2 M K^+ ions to the wild-type enzyme resulted in a 10 °C increase in the thermal denaturation temperature. No significant increase was observed in E98D or E98S. The lack of a significant effect of monovalent cations on the stability of E98D and E98S indicates that this alteration of the binding site eliminates cation binding. The thermal denaturation temperature of E98Q was 3 °C higher than that of the wild-type enzyme in the absence of the cation, indicating that the removal of the unbalanced, buried charge of Glu98 stabilizes the enzyme. These results confirm that Glu98 is a crucial residue in the interaction of monovalent cations with FTHFS.

Formyltetrahydrofolate synthetase (EC 6.3.4.3) catalyzes the ATP-dependent activation of formate ion via its addition to the N10 position of tetrahydrofolate in the reaction

$$\text{MgATP} + \text{formate} + (-)\text{-H}_4\text{folate} \leftrightarrow \text{MgADP} + (-)\text{-}N^{10}\text{-formyl-H}_4\text{folate} + P_i$$

FTHFS[1] is a highly expressed, key enzyme in both the Wood−Ljungdahl pathway of autotrophic CO_2 fixation (acetogenesis) and the glycine synthase/reductase pathways of purinolysis (*1*−*3*). The key physiological role of this enzyme in acetogens is to catalyze the formylation of tetrahydrofolate (H_4folate), an initial step in the reduction of carbon dioxide and other one-carbon precursors to acetate (*2*, *3*). In the purinolytic organisms, the enzyme reaction is reversed, liberating formate from 10-formyltetrahydrofolate with concurrent production of ATP (*1*).

The enzyme, a homotetramer with a MW of 240 000, has been shown to obligately require monovalent cations, particularly the alkali metals, for maximal catalytic activity (*4*) as well as maximal thermal stability (*5*). The enzymes from *Clostridium cylindrosporum* and *Clostridium acidiurici*, both mesophilic purinolytic organisms, require monovalent cations to prevent the active tetramer from dissociating into inactive monomeric subunits (*6*−*9*). However, this is only true when the incubation temperature of the enzyme is raised

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[‡] The PDB files of the atomic coordinates of Cs^+ and K^+ complexes of N^{10}-formyltetrahydrofolate synthetase are deposited in the Protein Data Bank as entries 1fpm and 1fp7.

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[1] Abbreviations: FTHFS, formyltetrahydrofolate synthetase; THF, tetrahydrofolate; KMB, potassium maleate buffer; EDTA, ethylenediaminetetraacetic acid; F_o, observed structure factor; F_c, calculated structure factor; DSC, differential scanning calorimetry.

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above 30 °C (*10*). The enzyme from the thermophilic homoacetogen, *Moorella thermoacetica* (formerly called *Clostridium thermoaceticum*), is stabilized against thermal inactivation by these cations, but does not require them for maintenance of tetrameric structure (*11*, *12*). Electron microscopic analysis of the enzymes from *M. thermoacetica* and *C. cylindrosporum* indicates that subunit association of the tetrameric enzyme occurs through dimeric intermediates (*13*). The binding regions between monomers have been shown to differ from those between dimers (*13*−*16*). Monomers associate to form a tight dimer complex through a series of hydrophobic interactions involving β-strand 9, α-helix 9, and β-strand 24 of subunits A and D (*16*).

Studies of FTHFS catalysis showed a strong enhancement of catalytic activity by ammonium ions, manifested as a decrease in the K_m of formate with an increasing ammonium ion concentration. At saturating levels of ammonium ion, a 10-fold decrease in the K_m for formate has been reported with no effect on the V_{max} of the reaction. Enzymes isolated from both purinolytic organisms and acetogens show this effect (*6*, *12*). The efficacy of the particular cation in question seems to be, at least in part, a function of its individual ionic radius (*9*, *17*); it decreases in the following order: $NH_4^+ > K^+ = Rb^+ > Cs^+ > Na^+ = Li^+$. The impact of monovalent cations on catalysis by FTHFS is greatest for the ammonium ion with maximum activation at 2 mM, while maximum activation by potassium ion occurs at 20 mM (*12*). It appears that the main mechanism for increasing activity with monovalent cations is the enhancement of formate binding. At high formate concentrations, the activation of FTHFS by monovalent cations becomes insignificant, while under the standard assay conditions, which include 40 mM formate, the maximum activation is 3-fold (*12*).

The importance of monovalent cations in the activation of enzymes has been amply documented. Studies examining their impact on catalysis have shown that these cations often act as allosteric effectors (*18*, *19*). However, fewer studies have investigated the biophysical effects monovalent cations have on enzymes, particularly with regard to their potential to impart thermal stability (*20*−*23*). In this study, we used X-ray crystallography to analyze the three-dimensional structures of cesium, potassium, and ammonium complexes of FTHFS. The high electron density of the cesium ion allowed us to unambiguously locate its binding site. However, upon examination of the structures of FTHFS−K^+ and FTHFS−NH_4^+ complexes, it was found that the cesium ion-binding site differed from that of the physiological cation-binding site. We tested this tentative assignment through site-directed mutagenesis of the Glu98 residue of FTHFS and subsequent analysis of the mutant enzymes through differential scanning calorimetry.

MATERIALS AND METHODS

Expression of M. thermoacetica FTHFS in Escherichia coli. The FTHFS structural gene was subcloned from pCRL47 S2A (*15*) into the phagemid, pAlter-1 (Promega), and transformed into *E. coli* strain JM109. This phagemid enables selection of transformants on the basis of resistance of the transformed cells to ampicillin. The subclone, designated MTFS-1 (*M. thermoacetica* FTHFS), expressed FTHFS at levels similar to those from the parent construct (data not shown).

Site Specific Mutagenesis of MTFS-1. Site specific mutations in the FTHFS gene contained in pAlter were introduced using and the Altered Sites II *in vitro* Mutagenesis System (Promega) (*24*). Oligonucleotides containing mismatches were synthesized by IDT Technologies (Coralville, IA). Mutants differing from the wild type and from each other at the same position, Glu98, were constructed. The native codon, GAG, was changed to CAG, GAC, and TCG to produce Gln98, Asp98, and Ser98, designated E98Q, E98D, and E98S, respectively. The mutations were confirmed by DNA sequencing.

Purification of FTHFSs from MTFS-1. Wild-type and mutant FTHFS enzymes were purified using a modification of the liquid chromatographic procedure of Staben (*25*), which takes advantage of the fortuitous binding of FTHFS to heparin. MTFS-1 and mutant crude extracts were prepared from 1 L cultures grown in Luria-Bertani medium (*26*) supplemented with 2.5 g/L dextrose, 2.5 g/L K_2HPO_4, and 10 μg/mL tetracycline (MTFS-1) or 100 μg/mL ampicillin (mutants). The cells were grown to early stationary phase and harvested by centrifugation. They were washed with 50 mM potassium maleate buffer (KMB) (pH 7.0), suspended in the same buffer, and disrupted by sonication using a Braun 5000 sonicator. All subsequent purification steps were performed at 4 °C. Cell debris was separated from the clear yellow supernatant by centrifugation for 40 min at 15000*g*. Streptomycin sulfate (4 mg/mL) was added to the supernatant, and the mixture was incubated with gentle stirring at 4 °C for 30 min (*27*). Nucleic acids and some contaminating protein were removed by centrifugation at 15000*g* for 40 min. The resulting crude extract was loaded onto a 15 cm × 1.5 cm heparin−agarose (Sigma) column pre-equilibrated with KMB. The column was washed with 30 mL of KMB, and proteins were eluted with a 0 to 0.8 M gradient of KCl in KMB. Fractions were assayed for activity (*12*), and those containing the greatest activities were pooled. Solid ammonium sulfate was added to a final concentration of 0.7 M. This solution was loaded onto a 15 cm × 1.5 cm phenyl-Sepharose (Sigma) column pre-equilibrated with KMB containing 0.7 M $(NH_4)_2SO_4$. The column was washed with 30 mL of equilibration buffer and eluted with a negative $(NH_4)_2SO_4$ gradient (0.7 to 0 M) in KMB. Fractions containing the highest activities were pooled, and SDS−PAGE chromatography was used to assess purity (*28*). Protein concentrations were assayed using the method of Lowry (*29*).

Crystallization and Crystal Soaking. Crystals of FTHFS were grown as described previously (*30*) by vapor diffusion from 46% saturated ammonium sulfate, 1 mM dithiothreitol, and 1% polyethylene glycol 1000, in 50 mM KMB (pH 7.6). The crystals belong to space group *R*32 and have the following unit cell dimensions: $a = 160.34$ Å and $c = 255.33$ Å with two subunits per asymmetric part of the unit cell. To exchange ammonium ions with cesium ions, FTHFS crystals were transferred from mother liquor to a 100 μL solution containing 53% saturated Cs_2SO_4 in 50 mM KMB (pH 7.6) and soaked overnight. An analogous procedure was used to obtain the potassium complex.

X-ray Data Collection, Processing, and Refinement. The FTHFS−Cs^+ crystals were cryoconditioned by soaking in 20% glycerol-enriched mother liquor. The data were collected from a single crystal flash-frozen at 100 K with R-Axis

Table 1: Crystallographic Data Collection and Refinement Statistics

	cesium complex	potassium complex
resolution range (Å)	20−3.0	20−3.2
total no. of reflections	137437	101296
no. of unique reflections	25052	20846
completeness (%)	97	99
mosaicity (deg)	1.1	1.3
R_{merge} (%)	8.8	11.6
Refinement Statistics		
R_{free} (%)	31.5	35.68
$R_{working}$ (%)	23.1	25.2
average *B*-factor (Å^2)	38.2	35.2
rmsd for bonds (Å)	0.0097	0.0085
rmsd for angles (deg)	1.4	1.5
no. of non-hydrogen protein atoms	8264	8264
no. of water molecules	270	270
no. of sulfate ions	11	11
no. of monovalent cations	2	2

IV mounted on a Rigaku RU-200 rotating anode source operating at 50 kV and 100 mA. The crystal-to-detector distance was 120 mm, and exposures were 20 min per 1.0° oscillation.

FTHFS−K^+ data were collected from a single crystal at the Structural Biology Center beamline at the Advanced Photon Source (Argonne National Laboratory, Argonne, IL). All data were integrated and scaled using the HKL2000 package (*31*). The free *R*-factors were used to justify each refinement step with 10% of the reflections set aside for R_{free} calculations. An energy minimization and isotropic temperature factor refinement implemented in the CNS package (*32*) was used for structure optimization. The relatively high crystallographic *R*-factors are most likely due to the absorption and the high density of the solvents. Actually, the crystals of FTHFS float in the cesium sulfate solution we used. Table 1 contains the summarized statistics of crystallographic data.

Differential Scanning Calorimetry. Pure FTHFS from MTFS-1 and mutants were concentrated using Centprep-30 concentrators (Amicon) and dialyzed against 50 mM PIPES buffered with tetramethylammonium hydroxide (pH 6.5) for 20 h to remove functional cations. The ratio of enzyme concentrate to dialysis buffer was 1:2000 (v:v), and the buffer was changed once after dialysis for 10 h. Using approximately 2.5 mg of dialyzed concentrated enzyme, power compensation differential scanning calorimetry (DSC) was conducted using a Calorimetry Sciences Corp. (Spanish Fork, UT) differential scanning calorimeter. Three trials were conducted for each enzyme: dialyzed enzyme, enzyme in the presence of 0.2 M KCl, and enzyme in the presence of 0.2 M KCl with 0.04 M formate. Formate was included on the basis of the observed decrease in the K_m for formate that results from monovalent cation binding (*12*).

RESULTS

Structure of FTHFS−Cs^+ and −K^+ Complexes. Difference Fourier maps were calculated with $|F_o| - |F_c|$ coefficients using the model from the original structure of FTHFS, determined in the presence of ammonium cation, which has been refined at 2.5 Å resolution (*16*). For the cesium ion complex, the resulting electron density map exhibited two strong peaks (14.6σ and 12.1σ for subunits A and B, respectively), which we assigned to cesium ions. The environment of the cesium peak in subunit A is shown in Figure 1. The binding site in the other subunit is very similar. The cesium cation is in contact with OE1 of Glu98 and the carbonyl oxygen atoms of Pro99, Ile125, Asn126, and Leu127. These ligands form an unbalanced polyhedron; we expect the presence of one or two water molecules at the cesium ion but in alternating locations and with their electron density blurred out by disorder. The protein residues forming the cesium ion-binding site belong to two coils; the first coil connects helices 5 and 6 in domain 1, while the other coil links β-strand 5 and α-helix 4 in domain 2. As shown in Figure 2, this part of the molecule is approximately at the interface between the two more tightly bound monomers. The cation ligands, however, are all from the same subunit; thus, the cation is not involved in cross-linking to the other subunit.

Unexpectedly, the structure of the ammonium ion complex (*16*) did not have an electron density peak at the cesium ion-binding site. Such a peak would likely represent an ammonium ion bound in this site. There were, however, three peaks at the carboxylate of Glu98, which were initially interpreted as water molecules. Since there is no other counterion in the vicinity of the buried side chain of Glu98, it is likely that one of the peaks actually represents a bound ammonium ion (Figure 3). This hypothesis was consistent with the structure of the FTHFS−K^+ complex. A difference

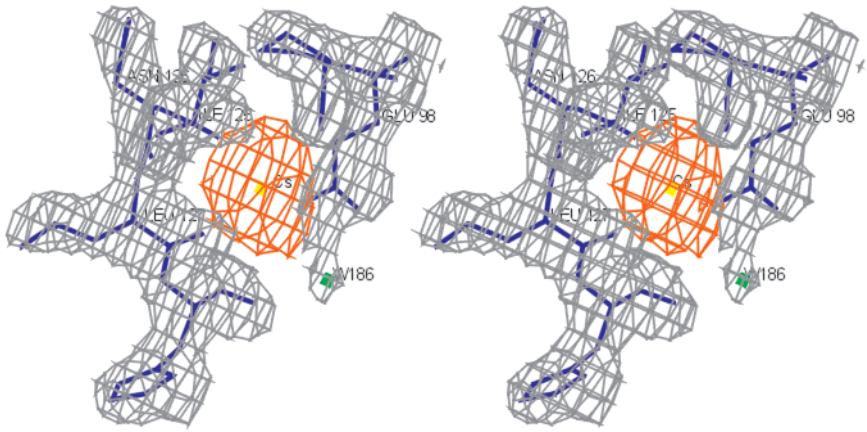

FIGURE 1: Stereodiagram of the difference Fourier map for the Cs^+ complex of FTHFS, calculated with $|F_o| - |F_c|$ coefficients and phased with the native FTHFS model. The basket contours, in orange, are at a 3σ level and show one of the cesium peaks. The $2|F_o| - |F_c|$ map shown for the rest of the model is shown in black at the 1.2σ level.

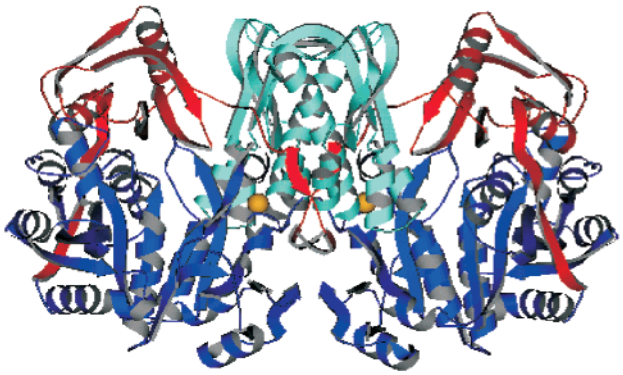

FIGURE 2: Positions of the monovalent cation binding sites in the tight dimer of FTHFS shown as yellow spheres. The three domains of each subunit are color coded with the N-terminus in blue and the C-terminus in red.

electron density map had the strongest peak, 7.8σ (5.2σ in the other subunit), between Glu98 and Asp132 (Figure 4); the peak is 7 Å from the position of Cs^+. The other ligands of K^+ are likely to be water molecules; however, even in the refined structure, we were unable to determine their positions due to disorder.

The location of the binding site of K^+ does not unequivocally identify the location of the ammonium ion. This is likely due to the difference in the preferred coordination geometry of these two cations; the ammonium ion tends to form four hydrogen bonds (*33*), while the potassium ion tends to have a coordination number of six or higher (*34*). Out of three peaks at Glu98 (Figure 5), the one assigned to water W186 is unlikely to represent ammonium ion. Its neighbors are as follows: OE2 of Glu98 at 2.8 Å, O of His12 at 3.0 Å, and N of Ile133 at 3.2 Å pointing with its H atom at W186. Thus, it appears that W186 is the donor of two H-bonds and the acceptor of one. The second water molecule, W270, is 2.3 Å from the K^+ binding site, and its neighbors are as follows: OE1 of Glu98 at 2.8 Å, OD1 of Asp132 at 3.2 Å, OG1 of Thr130 at 2.8 Å, and N of Thr130 at 2.7 Å, pointing with its H atom into the water molecule. Thus again, this environment is more suitable for a water molecule, a hydrogen bond acceptor, than for an ammonium ion. The last "water", W43, is, in our judgment, the most likely site of ammonium ion binding. Its position is the closest to the K^+ binding site, 1.7 Å, and it coordinates to OE1 of Glu98 at 2.8 Å and to OD1 of Asp132 at 2.9 Å. All other atoms are $\geq$3.5 Å away. Although the usual coordination number of the ammonium ion is higher, such a low coordination number is consistent with a moderate binding affinity (i.e., dissociation constants in the millimolar range), expected for a regulatory ligand such as potassium or ammonium ions.

Purification and Characterization of Mutant FTHFS. Preparations of wild-type enzyme (Glu98) exhibited specific activities (Table 2) equivalent to those seen in prior studies (*15*). The specific activity of the E98Q variant was similar to this as well. Replacement of K^+ and NH_4^+ ions in the assay mixture with Na^+ ions reduced the activity of the wild-type enzyme by 15%, while there was no significant change for the E98Q mutant. Specific activities of the E98D and E98S enzymes were significantly lower than that of either the wild-type or E98Q enzyme. Additionally, E98D purifications consistently yielded lower quantities of enzyme from the same amount of starting material than any other prepara-

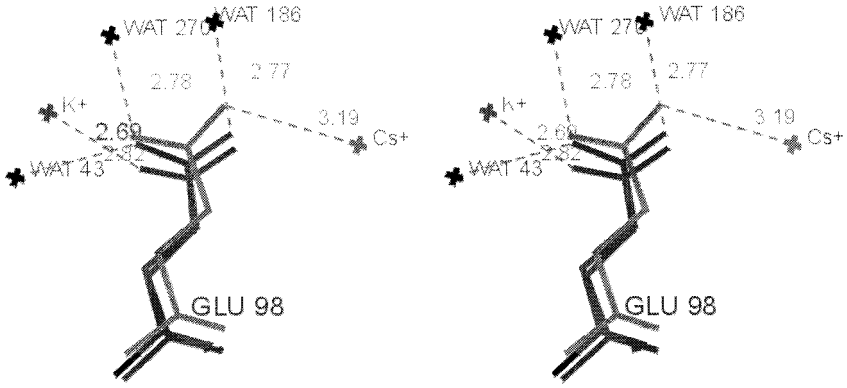

FIGURE 3: Superposition of Glu98 from the native, potassium, and cesium structures and the positions of cesium ion, potassium ion, and water molecules 43, 186, and 270.

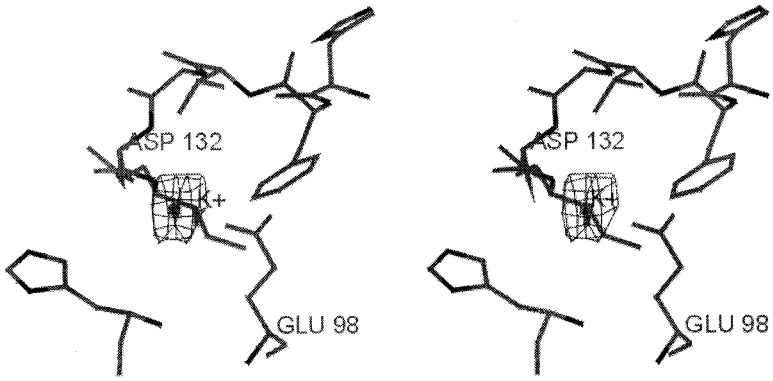

FIGURE 4: Physiological cation binding site. The calculated difference Fourier map for the K^+ complex is contoured at a 3σ level. The model is from the NH_4^+ complex of FTHFS.

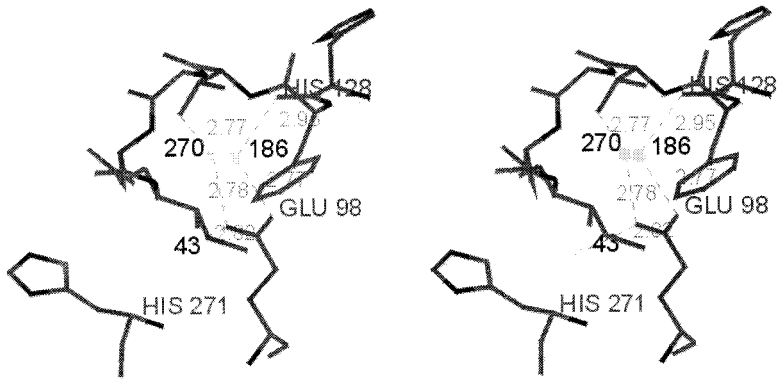

FIGURE 5: Out of the two water molecules present in the vicinity of K^+, W43 appears to be the more likely site of the ammonium ion.

Table 2: Specific Activities of Pure Preparations of Wild-Type and Mutant FTHFS's Determined as Described in ref *12*[a]

enzyme	specific activity [μmol of product min^{-1} (mg of protein)$^{-1}$] (percent of wild-type activity)
wild-type	780 (100)
E98Q	724 (93)
E98D	283 (36)
E98S	477 (61)

[a] Briefly, the prereaction mixture was 20 mM potassium maleate (pH 8.0), 1 mM NH_4Cl, 5 mM $MgCl_2$, 5 mM ATP, 40 mM formate, and 2 mM tetrahydrofolate.

Table 3: Results of Differential Scanning Calorimetry for Wild-Type and Mutant FTHFS's in the Absence and Presence of Potassium Ions and in the Presence of Potassium and Formate Ions[a]

	excess specific heat maximum (°C)		
	no bound cation	0.2 M KCl	0.2 M KCl and 0.04 mM formate
wild-type	69	79	79
E98Q	72	77	77
E98D	67	68	69
E98S	67	67	68

[a] Samples (0.5 mL) containing 2.40−3.35 mg of native or mutant enzyme in a 0.05 M PIPES/tetramethylammonium mixture (pH 6.5) with KCl and Na formate as shown were heated at a rate of 1 °C/min in sealed cups to 95 °C and then allowed to cool 1−5 h until reheating to 95 °C to obtain a baseline.

tion. Otherwise, the chromatograms from heparin−agarose of all four enzymes were identical, with each eluting at approximately 0.2 M KCl (data not shown). Phenyl-Sepharose chromatograms were also identical for all of the enzymes, all of which eluted at close to 0 M $(NH_4)_2SO_4$ (data not shown). Overloaded SDS−PAGE showed only one very intense band at a MW of 60 000 for all enzyme preparations.

Differential Scanning Calorimetry. Pure, concentrated wild-type and mutant enzymes were extensively dialyzed against tetramethylammonium hydroxide-buffered PIPES (see Materials and Methods). It was assumed at this point that the enzyme was essentially free of any contaminating potassium or ammonium ions. Each of the deionized enzyme preparations was examined using DSC. The wild-type enzyme exhibited a marked increase of ~10 °C in thermal stability upon addition of KCl (Table 3). While addition of K^+ ion to the E98Q enzyme increased the thermal stability by only 5 °C, the E98Q enzyme without potassium ion exhibited a 3 °C greater thermal stability relative to that of wild-type enzyme. Addition of potassium ions or potassium and formate ions had little stabilizing effect on the E98D enzyme. There was no stabilizing effect of potassium ions on the E98S enzyme and a negligible increase in stability in the presence of both potassium and formate ions. Addition of formate had no significant effect on the thermostability of any enzyme. However, it is not known whether the substrates bind in an ordered fashion. If formate binding requires prior binding of ATP and/or THF, the lack of thermal stabilization by formate would be simply due to the lack of binding.

The thermal denaturation profile of the E98D enzyme was smaller and relatively poorly resolved, so the excess heat capacity maxima (a measure of relative thermal stabilities) values were essentially indistinguishable. It is possible that a significant fraction of the E98D mutant is misfolded. However, even if only 36% (Table 2) of the protein were stabilized by potassium ions, a 5 or 10 °C shift would have been observed; hence, we believe that no significant stabilization of the E98D enzyme by potassium or formate ions occurs (see the Discussion). With the other mutants and the wild-type enzyme, a shift in the excess heat capacity maxima of as little as 2 °C is significant. In an extensive DSC study of another enzyme, enolase from yeast, the average standard deviation in T_{max} values was 0.6 °C in replicate samples (J. M. Brewer and J. E. Wampler, manuscript in preparation), so differences in T_{max} values of 1 °C are not considered significant.

DISCUSSION

Nearly one-third of all known enzymes require the presence of metal ions for catalytic activity. In metal-activated enzymes, the cations, which are usually the alkali or alkaline earth metal ions, are loosely bound to the enzyme. Divalent metal ions, especially those with a small ionic radius, often polarize substrates promoting some particular chemistry. Monovalent cations have only weak polarizing abilities and usually have a role in enzyme regulation. The results from this study also support the concept that in FTHFS monovalent cations are regulatory factors with respect to thermal stability. The cation-binding site is buried and far from the putative active site (*16*).

Most telling are the results obtained with the mutants at position 98. The lack of enzyme stabilization by potassium ion observed for E98D and E98S strongly suggests that there is no significant potassium ion binding to these mutants. Modeling (not shown) suggests that indeed in E98S and E98D chelation of a metal ion between Asp132 and residue

98 cannot take place. Additional mutation of Ile270 to a residue with a smaller side chain might allow for a different conformation of the Glu98 side chain and restoration of cation binding in E98D.

There is some stabilization by potassium ion observed for E98Q, which indicates cation binding. To balance the buried local charges when a monovalent cation binds between Gln98 and Asp132, it is possible that the side chain of Lys42, which forms an ion pair with Asp132, may move away to nearby Glu34 or Asp253. On the other hand, in E98Q with no cation bound there is no buried charge; this is in agreement with the observed thermal stability of E98Q being higher than that of wild-type FTHFS in the absence of activating monovalent cations.

Finally, the non-cation-binding mutants are quite active. Specific activity measurements for E98D and E98S showed only a 64 and 39% decrease in activity relative to that of the wild-type enzyme, respectively. In higher organisms, the S domain of trifunctional C1-folate synthase, whose sequence is 47% identical to those of the bacterial enzymes, carries out the FTHFS activity. The S domain has a glutamine in the place of Glu98, while Asp132 is conserved; thus, the E98Q mutant may be considered a reasonable electrostatic model for the mammalian enzyme.

FTHFS regulation by cations is effected mostly via tetramer stabilization. Two mechanisms can be envisioned. In the first, which could be labeled conformational, cation binding induces changes in the structure that propagate to the subunit interface and promote oligomerization. In the other, which could be termed electrostatic, the subunits remain rigid but the cation binding reduces the extent of repulsion between negatively charged macromolecules. Obviously, varying combinations of these mechanisms may take place in FTHFS's from different organisms. The studies reported here suggest that at least in this FTHFS the electrostatic mechanism dominates. Structure comparisons between FTHFS complexes with ammonium and cesium ions revealed that even though the cesium and the ammonium cations bind in different sites there are only minor structural differences between them. Main chain superposition of residues 98−101 and 124−132 of the cesium and potassium ion complexes on the ammonium ion complex showed only small differences (rmsd of 0.25 Å for cesium and 0.17 Å for potassium ion complexes), which appear to be insignificant. Also, the E98Q mutant without added potassium ion, in which an ion pair, carboxylate K^+, is replaced with neutral moieties, amide water, gains thermal stability. This supports the idea that no highly specific changes in intersubunit interactions are required, arguing against the conformational mechanism.

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