Crystal Structures of Chicken Liver Dihydrofolate Reductase: Binary ThioNADP$^+$ and Ternary ThioNADP$^+$·Biopterin Complexes†

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ABSTRACT: The role of the 3′-carboxamide substituent of NADPH in the reduction of pteridine substrates as catalyzed by dihydrofolate reductase (EC 1.5.1.3, DHFR) has been investigated by determining crystal structures at 2.3 Å of chicken liver DHFR in a binary complex with oxidized thionicotinamide adenine dinucleotide (thioNADP$^+$) and in a ternary complex with thioNADP$^+$ and biopterin. These structures are isomorphous with those previously reported for chicken liver DHFR [Volz, K. W., Matthews, D. A., Alden, R. A., Freer, S. T., Hansch, C., Kaufman, B. T., & Kraut, J. (1982) *J. Biol. Chem. 257*, 2528–2536]. ThioNADPH, which has a 3′-carbothioamide substituent in place of a 3′-carboxamide, functions very poorly as a coenzyme for DHFR [Williams, T. J., Lee, T. K., & Dunlap, R. B. (1977) *Arch. Biochem. Biophys. 181*, 569–579; Stone, S. R., Mark, A., & Morrison, J. F. (1984) *Biochemistry 23*, 4340–4346]. Comparisons show that, while NADP$^+$ and NADPH bind to DHFR with the pyridine ring and 3′-carboxamide coplanar, the thioamide group is twisted by 23° from the pyridine plane in both the binary and ternary complexes. This twist appears to be due to steric conflict between the thioamide sulfur atom and both the pyridine ring at C4 and the adjacent protein backbone at Ala-9. It results in an unfavorably close contact between the sulfur and the biopterin pteridine ring (0.9 Å less than the van der Waals separation) which, on the basis of the refined structure, greatly destabilizes the binding of biopterin. We infer that the thioamide in this position interferes with the transfer of a hydride ion from thioNADPH to the pteridine substrate by impeding movement of the coenzyme pyridine ring and substrate pteridine ring toward one another in the transition state. Thus, the planar conformation of the nicotinamide of NADPH when bound to DHFR appears to minimize steric repulsion during substrate binding to the holoenzyme complex and during transition-state formation.

Insight into the nature of an enzymic transition state can often be obtained by comparing the structure and catalytic behavior of the enzyme in complex with its substrates to those same properties of complexes with substrate analogs toward which the enzyme has significantly altered activity. In the case of dihydrofolate reductase (5,6,7,8-tetrahydrofolate: NADP$^+$ oxidoreductase, EC 1.5.1.3, DHFR), the enzyme responsible for catalyzing the NADPH-dependent reduction of 7,8-dihydrofolate and 7,8-dihydrobiopterin to their tetrahydro forms (Blakely, 1984; Freisheim & Matthews, 1984; Kraut & Matthews, 1987), studies with analogs of NADPH have shown that modification of the 3′-carboxamide of the nicotinamide ring greatly decreases catalytic activity (Williams et al., 1977; Dunn et al., 1977; Birdsall et al., 1980a,b, 1981; Stone et al., 1984). Thionicotinamide adenine dinucleotide phosphate (thioNADPH[1]), in which the carboxamide oxygen is replaced by sulfur, is one of the least active cofactor analogs, having only 3.6% the maximal activity of NADPH with chicken liver DHFR (B. T. Kaufman, unpublished results), 0.4% the maximal activity of NADPH with *Lactobacillus casei* DHFR (Williams et al., 1977), and essentially no activity with *Escherichia coli* DHFR (Stone et al., 1984).

Considering how seemingly small a change is involved in substituting a sulfur atom for a carboxamide oxygen, such a dramatic effect on the enzyme activity is surprising. Is the low activity of thioNADPH due to steric factors stemming from the larger van der Waals radius of the sulfur atom, or might it reflect purely electronic effects? That the cause might be a nonproductive binding geometry at the thionicotinamide group is suggested by *ab initio* calculations showing that the transition-state energy in hydride-transfer reactions of nicotinamide is substantially affected by the dihedral angle between the 3-carboxamide group and the pyridine ring (Donkersloot & Buck, 1981; Cummins & Gready, 1990).

The precise nature of thioNADPH binding to DHFR is also of interest since thioNADPH has been used as an inactive surrogate for NADPH in experiments aimed at quantifying the kinetics of dihydrofolate binding to the holoenzyme complex of *E. coli* (Fierke et al., 1987), *L. casei* DHFR (Andrews et al., 1989), and mouse DHFR (Thillet et al., 1990). It was implicitly assumed in these experiments that bound thioNADPH and bound NADPH affect substrate binding in the same way. In any case, thioNADPH was clearly the best available choice for a ligand that would occupy the cofactor binding site but prevent the forward reaction from proceeding, thereby permitting the measurement of association and dissociation rate constants for dihydrofolate.

We report here crystal structures of chicken liver DHFR, determined at a resolution of 2.3 Å, both as the binary

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[1] Abbreviations: DHFR, dihydrofolate reductase; cDHFR, chicken liver DHFR; NADP(+/H), nicotinamide adenine dinucleotide phosphate (oxidized/reduced); thioNADP(+/H), thionicotinamide adenine dinucleotide phosphate (oxidized/reduced); F_o, observed structure factor; F_c, calculated structure factor; α_c, calculated phases; *B*-factor, atomic temperature factor; rms, root mean square.

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FIGURE 1: Nicotinamide binding site in cDHFR·NADPH minus cDHFR·thioNADP$^+$ difference map contoured at $\pm 4\sigma$. Negative contours are depicted as dashed lines. Water molecules are depicted as spheres.

thioNADP$^+$ complex and as the ternary complex with thioNADP$^+$ and biopterin.[2] Fully oxidized biopterin, which has low but measurable activity with chicken liver DHFR (Kaufman, 1967), was used in these crystallographic studies instead of the more active dihydro form because of its much greater stability (Blakey, 1969).

MATERIALS AND METHODS

The ThioNADP$^+$ Binary Complex. Dihydrofolate reductase was purified from chicken livers as previously described (Kaufman & Kemerer, 1977). Crystals were grown by a procedure similar to that used for crystallization of the cDHFR·NADPH complex (Volz et al., 1982). A 200-μL aliquot of a solution containing 15 mg/mL cDHFR, 2 mM thioNADP$^+$ (Sigma Chemical), 10 mM calcium acetate, 2 mM dithiothreitol, and 20 mM Tris-HCl buffered at pH 7.5 was put in a 1-mL beaker, which was placed on a platform suspended inside a glass dish above 15 mL of a reservoir solution containing 23% (v/v) ethanol, 10 mM calcium acetate, and 20 mM Tris-HCl buffered at pH 7.5. The crystallization dish was covered, and the protein solution was allowed to equilibrate with the reservoir solution by vapor diffusion at 4 °C. Large crystals ($\sim 1.2 \times 0.5 \times 0.3$ mm) belonging to space group $C2$ with $a = 89.0$ Å, $b = 48.2$ Å, $c = 64.1$ Å, and $\beta = 124.6°$ appeared within 3 days. Crystals chosen for data collection were rinsed in a solution containing 40% ethanol, 10 mM calcium acetate, 2 mM thioNADP$^+$, and 20 mM Tris-HCl buffered at pH 7.5 before they were mounted in glass capillaries. A set of reflection intensities comprising 24 984 observations of 8627 unique reflections was collected on one crystal at ambient temperature ($\sim$20 °C) using the UCSD Multiwire Area Detector (Cork et al., 1973; Xuong et al., 1985). After the data were processed by locally developed programs (Anderson, 1987), the R_{sym}[3] was 0.03 for a data set containing 87% of all unique reflections to a Bragg spacing of 2.3 Å.

A difference Fourier was calculated with coefficients [F_o(thioNADP$^+$) – F_o(NADPH)] and α_c using the observed structure factors (F_o(NADPH)) and phases (α_c) from the refined 1.8-Å structure of cDHFR in binary complex with NADPH (S. J. Oatley, unpublished results; Brookhaven PDB entry 8DFR) to identify differences between the two complexes. The most significant features of this difference map (shown in Figure 1) are a positive and a negative peak (8σ) near the carboxamide moiety of NADPH. The negative peak, coincident with the carboxamide oxygen atom, and the larger positive peak adjacent to it suggest that the thioamide sulfur atom occupies a position approximately 1 Å from the position of the carboxamide oxygen of NADPH. Negative difference density (4σ) coincident with water molecules 629 and 650, which bind near the nicotinamide carboxamide oxygen and occupy part of the empty pteridine binding site, implies that these water molecules are less ordered or absent in the thioNADP$^+$ complex. The difference map also contains negative density (6σ) at the position of the side-chain sulfur of C11. This negative density is probably due to the cysteine side chain having been oxidized to a sulfenic acid in the NADPH complex crystals whereas it is maintained in the reduced sulfhydryl state in the thioNADP$^+$ crystals by the presence of dithiothreitol.

Restrained least-squares structure refinement was performed on the San Diego Supercomputer Center Cray X-MP/48 with a local version of the program PROLSQ (Konnert, 1976; Hendrickson, 1981) that incorporates a correction for X-ray scattering by amorphous solvent according to the method of Bolin et al. (1982). Refinement was initiated using the existing 1.8-Å cDHFR holoenzyme complex structure with the nicotinamide ring and waters 629 and 650 removed. After two cycles of PROLSQ refinement, a difference Fourier map with coefficients ($F_o - F_c$), α_c was calculated, and the thionicotinamide group was modeled into the resulting difference electron density. As this difference density suggested that the thioamide group is not coplanar with the pyridine ring no such restraint was applied, but the pyridine ring and the thioamide group were individually restrained to planarity. Refinement continued to convergence with iterative cycles of model rebuilding and least-squares minimization. Solvent atoms were modeled into positive difference density peaks that were greater than 3σ and within 2.4–3.2 Å of a heteroatom with reasonable hydrogen-bonding geometry. Waters 629 and 650 were not included as no significant density was observed near their positions. After 22 cycles of PROLSQ refinement, the R factor[4] is 0.16 for all data to 2.3 Å, the root mean square (rms) deviation in bond lengths from their dictionary values is 0.013 Å, and the rms deviation of atoms

[2] Coordinates for these structures have been deposited with the Brookhaven National Protein Data Bank and were assigned the codes 1DR2 for the cDHFR·thioNADP$^+$ complex and 1DR3 for the cDHFR·thioNADP$^+$·biopterin complex.

[3] $R_{sym} = \sum_{hkl}(\sum_i|I_i - \bar{I}|/\sum_i I_i)$, where I_i is the intensity of observation i of reflection hkl or a symmetry-related reflection and $\bar{I}$ is the scaled mean intensity. The summation is over all measured reflections.

[4] $R = \sum_{hkl}|F_o - F_c|/\sum_{hkl}F_o$.

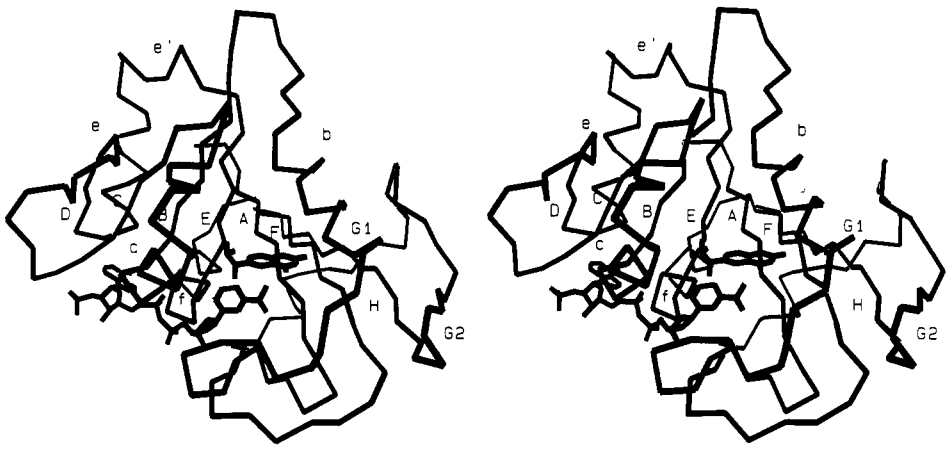

FIGURE 2: α-Carbon representation of chicken liver dihydrofolate reductase complexed with thioNADP+ and biopterin. β-strands are labeled with upper case letters and α-helices with lower case.

from their least-squares plane is 0.016 Å. These statistics correspond to a mean uncertainty in atomic positions of 0.15 Å, as estimated by the method of Luzatti (1952).

This final model includes 1469 protein, 48 thioNADP+, and 72 solvent atoms with average temperature factors of 33, 27, and 56 Å^2, respectively. The model also contains one calcium ion, required for crystallization but not for enzymic activity, that is located on the crystallographic 2-fold axis. Residues 187–189, a carboxy-terminal extension not found in other vertebrate DHFR sequences, remains unmodeled as no density was observed beyond V186. An additional 29 atoms in the side chains of residues R2, R28, E81, K84, E104, and K106 are also not included owing to absent or ambiguous density.

The ThioNADP+·Biopterin Ternary Complex. Crystals of the ternary complex were obtained by soaking previously grown crystals of the thioNADP+ binary complex in a solution containing 40% (v/v) ethanol, 20 mM Tris-HCl (pH 7.5), 10 mM calcium acetate, 2 mM dithiothreitol, 2 mM thioNADP+, and a saturating concentration of L-*erythro*-biopterin (Fluka Chemical) for 24 h. It is important to note that these are the same soaking conditions as had been used to obtain crystals of the ternary NADP+·biopterin complex (McTigue et al., 1992). The soaked crystals are isomorphous with the binary complex crystals, with unit cell dimensions a = 89.2 Å, b = 48.3 Å, c = 64.2 Å, and β = 124.8°. Data collection and reduction were performed as described for the binary complex. A data set containing 30 440 observations of 9286 unique reflections was collected from one crystal at ambient temperature. The R_{sym} is 0.03 and the data set is 91% complete to 2.3-Å resolution.

An initial model for least-squares refinement was obtained by modeling a biopterin molecule into positive electron density in a [F_o(thioNADP+·biopterin) − F_o(thioNADP+)], α_c difference Fourier, using phases from the refined thioNADP+ binary complex structure. After 31 cycles of PROLSQ refinement, the R factor is 0.14 for all data collected to 2.3-Å resolution, with deviations in bond lengths and planarity identical to those noted above for the binary thioNADP+ structure. These statistics correspond to an estimated mean uncertainty in atomic positions of 0.15 Å (Luzatti, 1952). The model contains 1490 protein, 48 thioNADP+, 17 biopterin, and 110 solvent atoms with average temperature factors of 37, 25, 56, and 57 Å^2, respectively. This structure, like that of the binary complex, also contains one calcium ion located on the crystallographic 2-fold axis. Atoms which remain unmodeled include residues 187–189 and 16 atoms in the side chains of residues R2, L80, E81, E104, and L178.

RESULTS AND DISCUSSION

Overall Protein Geometry. The structure of cDHFR in the complexes described here is essentially identical to that in the three other cDHFR complexes for which refined crystal structures are available: the NADPH binary complex (2.0 Å, Matthews et al. (1985a); 1.8 Å, S. J. Oatley unpublished results, Brookhaven PDB entry 8DFR), ternary complexes with NADPH and several inhibitors (Volz et al., 1982; Matthews et al., 1985a,b), and the ternary NADP+·biopterin complex (McTigue et al., 1992). After least-squares superpositioning with the program OVRLAP (Rossman & Argos, 1975), the rms deviation in equivalent Cα positions among these structures is less than 0.2 Å The largest difference in Cα positions between the binary thioNADP+ and ternary thioNADP+·biopterin complex structures is a 0.5–Å displacement at the carboxy-terminal V186. The folding of cDHFR, as well as the binding sites for thioNADP+ and biopterin, is shown in Figure 2. Of the 189 residues in cDHFR, 62 residues compose an eight-stranded β-sheet with seven parallel strands and a carboxy-terminal antiparallel strand. The remaining residues form five α-helices, one left-handed poly(proline) helix, eight tight turns, and five extended loops. As this folding pattern is identical to that of human DHFR in binary complex with folate, secondary structure assignments are the same as those given for that structure (Davies et al., 1990).

ThioNADP+ Binding. As seen in Figure 2, thioNADP+, like NADP+, binds to DHFR in an extended conformation with its adenine ring occupying a relatively solvent-exposed site near the edge of the β-sheet, while the nicotinamide ring is bound near the center of the molecule. A separation between the two central β-strands, βA (residues 4–10) and βE (residues 108–116), creates a cavity where the nicotinamide ring is bound approximately parallel to the β-sheet. As DHFR is an A-side-specific pyridine nucleotide-dependent enzyme, the B-face of the nicotinamide ring is sequestered by protein atoms while the A-face is directed toward the binding site for the dihydropteridine ring of substrate. Since the conformation of thioNADP+ is identical in the binary thioNADP+ and ternary thioNADP+·biopterin complexes, the following discussion refers to both complexes.

The largest difference between the binding geometry of thioNADP+ and either NADP+ or NADPH occurs at the thionicotinamide ring (see Figure 3). The thionicotinamide pyridine ring is located approximately 0.3 Å farther from the backbone strand βA as compared with the pyridine ring position in the structures of the NADPH and NADP+·biopterin

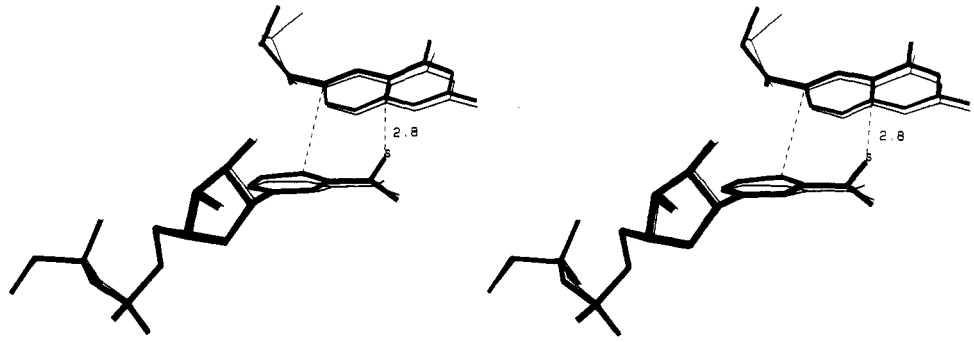

FIGURE 3: Coordinates of the thionicotinamide mononucleotide and pyrophosphate of thioNADP$^+$ and biopterin in the cDHFR·thioNADP$^+$·biopterin complex structure (thick lines) superpositioned with those of NADP$^+$ and biopterin in the cDHFR·NADP$^+$·biopterin complex structure (thin lines). The hydride-transfer route between C4 of the thionicotinamide and C6 of biopterin and the short 2.8-Å separation between the thioamide sulfur and the biopterin pteridine ring are indicated by dashed lines.

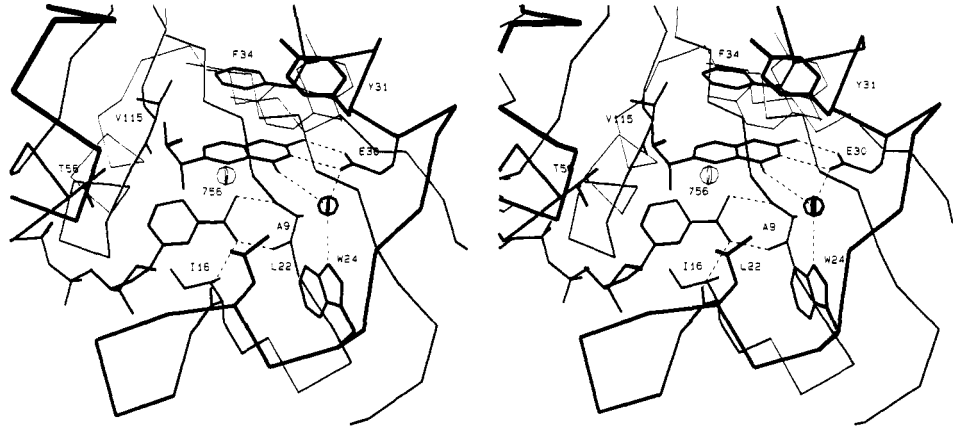

FIGURE 4: Catalytic site of the cDHFR·thioNADP$^+$·biopterin complex (thick lines). Hydrogen bonds are depicted as dashed lines and water molecules as spheres. For comparison, the two positions of the Y31 side chain and the position of water 756 in the cDHFR·NADP$^+$·biopterin complex are included (thin lines).

complexes of cDHFR (Figure 4). This difference is probably due to the increased length of the C=S bond (1.61 Å, average refined length in these complexes) as compared to the C=O bond (1.25 Å, refined length for the 3′-carboxamide substituent in the cDHFR·NADP$^+$·biopterin and cDHFR·NADPH complexes), which displaces the thionicotinamide group toward V115 to avoid unfavorably close contacts between the thioamide sulfur atom and the backbone of A9. In addition, the thioamide group is twisted approximately 23° relative to the pyridine plane (Figure 3) in both the thioNADP$^+$ and thioNADP$^+$·biopterin complexes, whereas in all known structures of DHFR containing bound NADPH or NADP$^+$ (Filman et al., 1982; Matthews et al., 1985a,b; Stammers et al., 1987; Bystroff et al., 1990; McTigue et al., 1992), the carboxamide group is coplanar with the pyridine ring and in the *trans* conformation (defined as τ(C4–C3–C7–O7) = 0°) *i.e.*, with O7 *syn* to C4. A twist of the thioamide with respect to the pyridine ring is not unexpected, however, as such a rotation is necessary to avoid an unfavorably close contact between the sulfur and the CH group at position 4 of the pyridine ring. In fact, in the crystal structure of 2-methyl-4-(thiocarbamoyl)-pyridine (2-methylisothionicotinamide), the thioamide is twisted out of the pyridine plane by 60° (Gadret & Gourselle, 1969). The 23° twist seen in these complexes with DHFR, therefore, most likely does not completely relieve the unfavorable interaction between the pyridine ring and the sulfur. However, a larger rotation of the thioamide group is forbidden by the protein geometry in the nicotinamide binding site.

The three hydrogen bonds between the 3′-carboxamide group of NADPH or NADP$^+$ and the backbone segment 9–16 in cDHFR are maintained in these thioNADP$^+$ complexes (shown in Figure 4). The thioamide NH_2 makes two hydrogen bonds to the the backbone carbonyl oxygens of the conserved residues A9 and I16. The thioamide sulfur is 3.3 Å from the backbone NH of A9, and the Cα(A9)–N(A9)–S7(thio-NADP$^+$) angle is 105°. This geometry probably represents an NH–S hydrogen bond, since theoretical calculations on hydrogen bonds involving NH_3 as a proton donor and H_2S as a proton acceptor predict a 4-Å separation between the nitrogen and sulfur atoms (Kollman et al., 1975), and apparent hydrogen bonds between amino acids and cysteine sulfhydryls typically have nitrogen to sulfur separations of between 3.4 and 3.7 Å in protein structures (Gregoret et al., 1991).

Although NMR studies of the *L. casei* DHFR·thioNADP$^+$ complex indicate that the adenosine and pyrophosphate groups of thioNADP$^+$ are bound within the enzyme molecule while the thionicotinamide group is flipped out of the cofactor binding crevice into solution (Hyde et al., 1980a,b; Feeney et al., 1983), in the cDHFR crystal structures described here the electron density for all portions of the thioNADP$^+$ molecule, including the thionicotinamide group (see Figure 5), is well defined in both the binary and ternary complexes. The average temperature factors for the nicotinamide group in the cDHFR·thioNADP$^+$ and cDHFR·thioNADP$^+$·biopterin structures are 25.2 and 24.1 Å^2, respectively. In fact, aside from the small differences noted above between the positions of the nicotinamide and thionicotinamide groups, the positions of the remainder of the coenzyme and enzyme are essentially identical in the NADPH, thioNADP$^+$, NADP$^+$·biopterin, and thioNADP$^+$·biopterin complexes of cDHFR. The protein–coenzyme interactions for these thioNADP$^+$ complexes are, therefore, the same as those described for the NADPH and

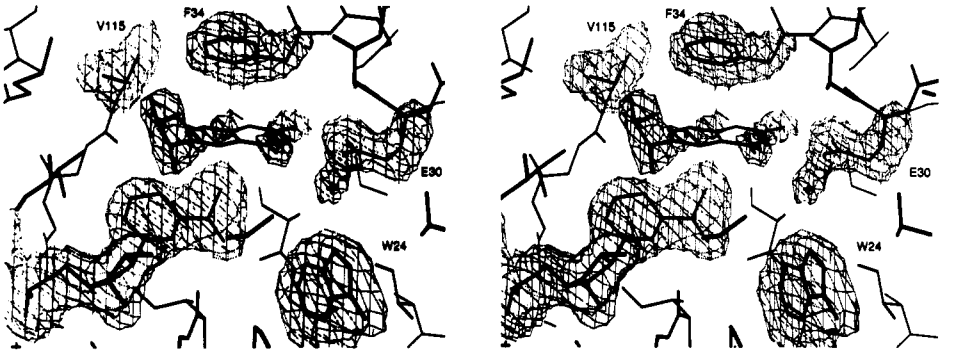

FIGURE 5: Omit map ($F_o - F_c$) showing the quality of the refined cDHFR·thioNADP$^+$·biopterin complex structure. The map was generated by removing from the model atoms in the side chains of W24, E30, F34, and V115, all atoms of the biopterin molecule, water 230, and all atoms of the nicotinamide mononucleotide and pyrophosphate groups of thioNADP$^+$. The density shown here is contoured at $+2.75\sigma$. At $+2.75\sigma$ the density for biopterin is fragmented but becomes continuous at a contour level of $+2.25\sigma$.

NADP$^+$·biopterin complexes of cDHFR (McTigue et al., 1992). Comparison of the *L. casei* DHFR·NADPH·methotrexate (Bolin et al., 1982; Filman et al., 1982), *E. coli* DHFR·NADP$^+$·folate (Bystroff et al., 1990), chicken DHFR·NADPH (S. J. Oatley, unpublished results) chicken DHFR·NADP$^+$·biopterin (McTigue et al., 1992), and human DHFR·folate (Davies et al., 1990) complexes shows that the structures of all of the nicotinamide binding sites are very similar. There is, therefore, no obvious structural reason why thioNADP$^+$ should not bind similarly to all of these enzymes.

Waters 629 and 650, which occupy part of the pteridine binding site in the 1.8-Å cDHFR·NADPH complex structure, are absent in the cDHFR·thioNADP$^+$ complex structure. The lack of significant density for waters at these positions in the thioNADP$^+$ complex is not surprising, as the temperature factors for these waters are greater than 60 Å^2 in the NADPH complex structure, and waters at these positions would sterically clash with the thioamide sulfur atom.

Biopterin Binding. The binding of biopterin is greatly disturbed by its interaction with bound thioNADP$^+$. Comparison of the structures of the NADP$^+$·biopterin (McTigue et al., 1992) and thioNADP$^+$·biopterin complexes of cDHFR (Figure 3) shows that the biopterin molecule in the latter is translated approximately 0.3 Å away from the nicotinamide binding site. Much more significant, however, is that the average temperature factor for biopterin has increased from 12 Å^2 in the NADP$^+$·biopterin complex (McTigue et al., 1992) to 57 Å^2 in the thioNADP$^+$·biopterin complex. Since the crystallization and soaking procedures used to obtain these two complex crystals were identical, as emphasized earlier, this difference in temperature factors for biopterin most likely represents a destabilization of biopterin binding in the thioNADP$^+$ ternary complex relative to biopterin binding in the NADP$^+$ ternary complex. Indeed, the unusually high temperature factors as well as other evidence (see below) suggests that the biopterin binding site may be only fractionally occupied in the ternary complex structure (see Figure 5). This destabilization is most likely due to a very unfavorable contact between the thioamide sulfur and C8A of the pteridine ring which, at 2.8 Å, is 0.9 Å less than the expected van der Waals separation (Bondi, 1964). By comparison, the corresponding atomic separation in the NADP$^+$·biopterin complex is 3.3 Å, as expected for a van der Waals contact between a carbonyl oxygen and an aromatic CH group. Additionally, the observations that waters 629 and 650, which occupy part of the pteridine binding site in the cDHFR·NADPH complex structure, are not present in the cDHFR·thioNADP$^+$ complex structure provides further evidence that the presence of the thioamide interferes with the binding of ligands in the pteridine binding site.

The apparent looseness of biopterin binding in the thioNADP$^+$·biopterin complex correlates with other details of the protein structure surrounding the pteridine binding site. That is to say, these details are seen to be intermediate between those of the NADPH binary complex and those of the NADP$^+$·biopterin ternary complex, as though the biopterin site were only fractionally occupied. In the NADPH binary complex of cDHFR, the side-chain carboxylate of E30, the acidic residue believed to be involved in protonation of the substrate (Howell et al., 1986), is disordered ($\langle B \rangle$ = 74 Å^2). In the NADP$^+$·biopterin complex, however, this carboxylate is significantly stabilized ($\langle B \rangle$ = 9 Å^2) by a pair of hydrogen bonds to the N3 and 2-amino groups of biopterin. By comparison, the average *B*-factor for this carboxylate is 39 Å^2 in the thioNADP$^+$·biopterin complex, even though the hydrogen bonds between the E30 side chain and the biopterin are also formed in this complex. In addition, the side chain of Y31 has only one conformation in the NADPH complex, but two distinct conformations in the NADP$^+$·biopterin complex. In the thioNADP$^+$·biopterin complex, this side chain assumes only one of these conformations, equivalent to that observed in the NADPH complex. And finally, an ordered water molecule (water 756) that is hydrogen bonded to O4, N5, and the two hydroxyl groups of biopterin in the NADP$^+$·biopterin complex is not observable in the thioNADP$^+$·biopterin complex. This water molecule, which is thought to occupy a site close to that of a transiently bound water involved in substrate protonation (McTigue et al., 1992), is probably not seen in the thioNADP$^+$·biopterin complex owing to disordering of the bound biopterin. Thus, both the elevated temperature factors for the biopterin itself and the above-mentioned features of its binding site indicate that biopterin is bound much less tightly in a ternary complex with thioNADP$^+$ than in a ternary complex with NADP$^+$.

Low Activity of ThioNADPH. These structures strongly suggest that the steady-state rate of dihydrofolate reduction when thioNADPH is used as the cofactor is limited not by product dissociation, as is the case with NADPH, but rather by impaired substrate binding and/or slow hydride transfer. With NADPH as cofactor, the rate of tetrahydrofolate dissociation limits the steady-state rate of dihydrofolate reduction, as catalyzed by *E. coli* (Fierke et al., 1987), *L. casei* (Andrews et al., 1989), and mouse (Thillet et al., 1990) DHFR, while for human DHFR the steady-state rate is limited by tetrahydrofolate and NADP$^+$ dissociation (Appleman et al., 1990). Destabilization of pteridine binding, as seen here

for biopterin, is also likely to occur for folate compounds since the pteridine rings in the crystal structures of the *E. coli* DHFR·NADP$^+$·folate (Bystroff et al., 1990) and cDHFR·NADP$^+$·biopterin complexes occupy nearly identical positions. In fact, in ternary complexes with either thioNADP$^+$ or thioNADPH and a tetrahydropteridine substrate, the crowding is likely to be even more severe than in the cDHFR·NADP$^+$·biopterin complex structure owing to a nonplanar conformation of the tetrahydropteridine ring. In the crystal structure of 5,6,7,8-tetrahydrobiopterin (Matsuura et al., 1985), C6 extends 0.6 Å out of the pteridine plane in a direction that would correspond to moving it even closer to the nicotinamide ring. While a close contact between C6 of the tetrahydropteridine and the nicotinamide rings is probably also responsible for the negative cooperativity in the binding of tetrahydrofolate and either NADP$^+$ or NADPH to DHFRs (Fierke et al., 1987, Andrews et al., 1989; Thillet et al., 1990; Appleman et al., 1990), the effect will probably be much larger for ternary complexes with thioNADP$^+$ or thioNADPH owing to the positioning and greater size of the thioamide group. Tetrahydrofolate release from either the ternary cDHFR·thioNADP$^+$·tetrahydrofolate or cDHFR·thioNADPH·tetrahydrofolate complexes is, therefore, probably much faster than from the corresponding complexes with NADP$^+$ or NADPH.

For a number of reasons, the position of the thioamide group, as seen in these crystal structures, may slow the transfer of a hydride ion from C4 of thionicotinamide to C6 of dihydropteridine substrates. Theoretical calculations on the transition state for reduction of methyleniminium cation by 1,4-dihydropyridine predict the separation between the hydride donor and acceptor atoms to be 2.6 Å (Wu & Houk, 1987). In the dead-end *E. coli* DHFR·NADP$^+$·folate and cDHFR·NADP$^+$· biopterin complex structures, the distance between the hydride donor atom (C4 of the nicotinamide) and the hydride acceptor (C7 of pteridine) is 3.1 and 3.3 Å, respectively, already about 0.5 Å shorter than the expected van der Waals contact distance. The pteridine and nicotinamide rings must, therefore, move still closer together from their already close ground-state positions to enter the transition state.

Previously determined DHFR complex structures have suggested that the desired 2.6-Å separation might be achieved by small rotations of both the nicotinamide and pteridine rings (Davies et al., 1990; McTigue et al., 1992). In particular, these structures suggest that the nicotinamide pyridine ring could rotate 9° about an axis collinear with the C2–C3 bond, moving C4 closer to the substrate pteridine ring while the carboxamide group remains fixed by three hydrogen bonds to backbone atoms of A9 and I16 (McTigue et al., 1992). Thus, as the transition state is approached, the nicotinamide pyridine ring would twist slightly relative to its carboxamide group. Such a rotation seems feasible since the planar *trans* conformation of 1-methyldihydronicotinamide and conformations in which the carboxamide group is rotated up to 30° from this conformation have approximately equal energies (Cummins & Gready, 1989). However, the same is unlikely to be true for thioNADPH. As noted above, thioNADPH is bound to DHFR with its thioamide group twisted with respect to the pyridine ring. Rotation of the pyridine ring toward the pteridine ring would, therefore, be disfavored as it would decrease the already short separation between the thioamide sulfur and C4 of the pyridine ring. Similarly, rotation of the pteridine ring toward the pyridine ring would also be disfavored as this movement would further decrease the already very close contact between the pteridine ring and the thioamide sulfur.

A similar steric interference by the thioamide sulfur is also predicted for a transition state involving a puckered dihydropyridine ring. Although the dihydronicotinamide ring appears planar in crystal structures of N-substituted dihydronicotinamide compounds (Karle, 1961; Glasfield et al., 1988) and in crystal structures of NADH or NADPH bound to enzymes (Filman et al., 1982; Volz et al., 1982; Matthews et al., 1985a,b; Schneider et al., 1985; Cedergren-Zeppezauer et al., 1985; Piontek et al., 1990), NMR studies on NADH indicate that the dihydronicotinamide ring exists in a boat conformation in solution (Oppenheimer et al., 1978), and some theoretical calculations predict that the dihydropyridine ring adopts a boat conformation in the transition state of model hydride-transfer reactions with a 1,4-dihydropyridine ring as the hydride donor (Wu & Houk, 1991). If, however, puckering of the dihydronicotinamide ring does indeed occur in the transition state of reactions catalyzed by DHFR, the same steric problems would be encountered. For a thionicotinamide ring bound with its thioamide twisted by 23° relative to the pyridine ring, transition from a planar to a boat conformation would *decrease* the separation between C4 (dihydropyridine) and S7 (thioamide) and result in further steric crowding. Thus, in summary, it appears that the larger size of the thioamide sulfur as compared to an oxygen atom may be sufficient to interfere with the small adjustments needed to attain the optimum transition-state geometry for hydride transfer, regardless of whether it involves a thionicotinamide ring in the planar or the boat conformation.

Another factor which may contribute to a lower rate of hydride transfer from thioNADPH than from NADPH is the difference in oxidation–reduction potentials between these dinucleotides. The oxidation–reduction potential of the thioNAD$^+$–thioNADH system is –0.285 V (Anderson & Kaplan, 1959), compared with –0.320 V for the NAD$^+$–NADH system (Burton & Wilson, 1953; Rodkey, 1955). Therefore, all other factors being equal, hydride transfer should be more favored from NADPH than from thioNADPH. However, as the oxidation–reduction potential of these groups may be significantly altered by binding to the enzyme, the actual effect on the hydride-transfer rate remains undetermined.

Catalytic Advantage of the Binding Conformation of the NADPH Nicotinamide. The structures presented here suggest why it is sterically advantageous for DHFR to bind the nicotinamide moiety with its 3′-carboxamide group and pyridine ring in the same plane: this conformation minimizes interference of substrate binding with the rotation of the pteridine ring toward the nicotinamide ring in the transition state. Although steric interference would be less pronounced with NADPH than with thioNADPH, owing to the shorter C=O bond as compared with a C=S bond and the smaller van der Waals radius of oxygen as compared to sulfur, a 20–30° twist of the carboxamide from the plane of the pyridine ring would cause the carboxamide oxygen to extend 0.2–0.3 Å farther into the pteridine binding site. However, as these same steric arguments would also apply to the planar *cis* conformation (defined as τ(C2–C3–C7–O7) = 180°), there must be some further advantage, possibly electronic, to binding the nicotinamide ring in the planar *trans* conformation. A *trans* orientation, with the carboxamide oxygen *syn* to C4 of the pyridine ring, is also observed for coenzymes bound to alcohol dehydrogenase (Eklund et al., 1984), lactate dehydrogenase (Grau et al., 1981; Piontek et al., 1990), glyceraldehyde-3-phosphate dehydrogenase (Moras et al., 1975; Biesecker et al., 1977; Skarżyński et al., 1987), and malate

dehydrogenases (Birktoft et al., 1989). For reactions in which the substrate is cationic in the transition state, as is believed to occur in the case of DHFR (Gready, 1985; Morrison & Stone, 1988), semiempirical and *ab initio* calculations predict that an electrostatic interaction between the cationic species and the nicotinamide carbonyl oxygen can significantly stabilize the transition state (Donkersloot & Buck, 1981; Cummins & Gready, 1990).

It is also reasonable to suppose that if, as described above, transition-state formation involves movement of the C4 of nicotinamide toward the substrate pteridine and a consequent twist of the carboxamide, this movement will occur more readily from a planar *trans* conformation than from the planar *cis* conformation. Theoretical calculations predict the *cis* conformation of nicotinamide and dihydronicotinamide to be an absolute energy minimum (Cummins & Gready, 1989), whereas the *trans* conformation and conformations with the carboxamide twisted by up to 30° have approximately equal energies. If these calculations are correct, binding the nicotinamide in a *cis* conformation in the ground state, as opposed to *trans*, would increase the energy difference between the ground state and the transition state by 1–3 kcal/mol (Cummins & Gready, 1989) for a transition state which has the carboxamide group twisted by approximately 30° with respect to the nicotinamide ring.

Substrate Binding to the Enzyme–NADPH Complex. Because of its low activity thioNADPH has been used in place of NADPH in the measurement of association and dissociation rate constants for dihydrofolate binding to the holoenzyme in the kinetic schemes for *E. coli* (Fierke et al., 1987), *L. casei* (Andrews et al., 1989), and mouse DHFR (Thillet et al., 1990). The disorder in biopterin binding in the structure of the cDHFR·thioNADP$^+$·biopterin complex structure as compared to the cDHFR·NADP$^+$·biopterin complex structure suggests that these kinetic measurements may not accurately reflect dihydrofolate binding to the NADPH–holoenzyme complex and that these rates should be redetermined by an alternative method. However, the effect is not expected to be large, probably a factor of 6 or less. This can be seen from the relatively small variation in these rate constants (×6.5 at most) found by Fierke et al. (1987) when the cofactor site of *E. coli* DHFR is alternately occupied by thioNADPH, NADP$^+$, or nothing but solvent. The reason is that only the dihydropteridine ring of the substrate is in contact with the cofactor; therefore, binding interactions of the *p*-aminobenzoyl glutamate tail will not be affected.

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