Role of the Active-Site Carboxylate in Dihydrofolate Reductase: Kinetic and Spectroscopic Studies of the Aspartate 26 → Asparagine Mutant of the *Lactobacillus casei* Enzyme†

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ABSTRACT: A mutant of *Lactobacillus casei* dihydrofolate reductase, D26N, in which the active site aspartic acid residue has been replaced by asparagine by oligonucleotide-directed mutagenesis has been studied by NMR and optical spectroscopy and its kinetic behavior characterized in detail. On the basis of comparisons of a large number of chemical shifts and NOEs, it is clear that there are only very slight structural differences between the methotrexate complexes of the wild-type and mutant enzymes and that these are restricted to the immediate environment of the substitution. The data suggest a slight difference in orientation of the pteridine ring in the binding site in the mutant enzyme. Both NMR and UV spectroscopy show that methotrexate is protonated on N1 when bound to the wild-type enzyme but not when bound to the mutant. Binding constant measurements by fluorescence quenching and steady-state kinetic measurements of dihydrofolate (FH_2) and folate reduction show that the substitution has little or no effect on substrate, coenzyme, and inhibitor binding (<7-fold increase in K_d) and only a modest effect on k_{cat} (up to a factor of 9 for FH_2 and 25 for folate) and k_{cat}/K_M (up to a factor of 13 for FH_2 and 14 for folate). Measurements of deuterium isotope effects and direct measurements of hydride ion transfer and product release by stopped-flow methods revealed that for the mutant enzyme hydride ion transfer is rate-limiting across the pH range 5−8. This allowed a direct comparison of the rate of hydride ion transfer in the wild-type and mutant enzymes; the asparagine substitution was found to decrease this rate by 62-fold at pH 5.5 and 9-fold at pH 7.5. This effect is much smaller than that seen for the corresponding mutant of *Escherichia coli* dihydrofolate reductase [Howell, E. E., Villafranca, J. E., Warren, M. S., Oatley, S. J., & Kraut, J. (1986) *Science 231*, 1123−1128], estimated as a 1000-fold decrease in the rate of hydride ion transfer. The change in pH dependence of k_{cat} resulting from the substitution is consistent with, but does not prove, the idea that the group of pK 6.0 which must be protonated for hydride ion transfer to occur is Asp26. For folate reduction, the pH dependence of k_{cat} is determined by two pKs, one of which, pK 5, disappears in the mutant enzyme, suggesting that it may correspond to ionization of Asp26. The second pK, 7.3 in both wild-type and 6.9 in the mutant enzyme, may be an ionization of the folate molecule. The small effect of the Asp26 → Asn substitution in *L. casei* dihydrofolate reductase makes it unlikely that this group acts as an essential proton donor in catalysis. The possible roles of Asp26 and Asn26 in catalysis are discussed.

Dihydrofolate reductase (dhfr) catalyzes the reduction of dihydrofolate (and, less effectively, folate) to tetrahydrofolate, using NADPH as coenzyme. It has been studied extensively over the years, both as a small dehydrogenase convenient for study by crystallography and NMR spectroscopy and as the target for a number of clinically important drugs such as methotrexate and trimethoprim. High-resolution crystal structures have been determined for dhfr from prokaryotic and eukaryotic organisms in a number of complexes with substrates and inhibitors such as methotrexate and trimethoprim (Bolin *et al.*, 1982; Filman *et al.*, 1982; Volz *et al.*, 1982; Matthews *et al.*, 1985a,b; Stammers *et al.*, 1987; Oefner *et al.*, 1988; Bystroff *et al.*, 1990; McTigue *et al.*, 1992, 1993). Building on this structural information, and that obtained by NMR spectroscopy [see, *e.g.*, Roberts (1990a,b) and Feeney (1990)], and on the knowledge of the kinetic mechanism of the enzyme (Fierke *et al.*, 1987; Andrews *et al.*, 1989; Thillet *et al.*, 1990), site-directed mutagenesis has been extensively used to elucidate the roles of individual amino-acid residues in substrate and inhibitor binding and catalysis (Villafranca *et al.*, 1983; Chen *et al.*, 1985, 1987; Howell *et al.*, 1986, 1987; Benkovic *et al.*, 1988; Prendergast *et al.*, 1988, 1989; Birdsall *et al.*, 1989a; Andrews *et al.*, 1989; Jimenez *et al.*, 1989; Warren *et al.*, 1991; David *et al.*, 1992; Li *et al.*, 1992; Wagner *et al.*, 1992; Dion *et al.*, 1993; Thomas *et al.*, 1994).

One residue which has attracted particular attention is the conserved carboxyl-containing residue in the active site—aspartic acid in all known bacterial dhfrs (Asp26 in the *Lactobacillus casei* enzyme[1]) and either aspartic acid

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[1] Unless otherwise noted, all residue numbers are those for *L. casei* dihydrofolate reductase.

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or glutamic acid in all other known dhfrs. Spectroscopic studies have shown that 2,4-diaminopteridine and 2,4-diaminopyrimidine inhibitors of dhfr are protonated on N1 when bound to the enzyme (Hood & Roberts, 1978; Cocco *et al.*, 1981; Roberts *et al.*, 1981; Birdsall *et al.*, 1989), and it is clear from the crystal structures of inhibitor complexes that there is an ion pair between N1 and the 2-amino group of the bound inhibitor and the carboxylate of this aspartate or glutamate [*e.g.*, Bolin *et al.* (1982)].

The substrates dihydrofolate (FH_2) and folate bind in a different orientation from the inhibitors (Hitchings & Roth, 1980; Charlton *et al.*, 1979, 1985), with N3 rather than N1 being close to the essential carboxylate (Oefner *et al.*, 1988; Bystroff *et al.*, 1990). It is widely assumed that the reduction of the N5−C6 bond of FH_2 is promoted by transfer of a proton to N5 (Huennekens & Scrimgeour, 1964; Gready, 1985). The pH dependence of hydride ion transfer indicates that it requires that a group with a pK of 6.0−6.5 be in the protonated state (Fierke *et al.*, 1987; Andrews *et al.*, 1989; Thillet *et al.*, 1990); this has been proposed to be the active-site carboxyl, since this is the only ionizable group of the enzyme close to the pteridine ring in the active site. The observation that substitution of Asp27 by asparagine in *Escherichia coli* dhfr leads to a 300-fold decrease in k_{cat} with little or no structural perturbation (Howell *et al.*, 1986) is entirely consistent with this idea. It has been further suggested (Matthews *et al.*, 1977; Hitchings & Roth, 1980; Freisheim & Matthews, 1984) that this carboxyl is the source of the proton which is transferred to N5 of FH_2. However, the mode of binding of folate (Oefner *et al.*, 1988; Bystroff *et al.*, 1990) and that proposed for FH_2 binding (Bolin *et al.*, 1982; Oefner *et al.*, 1988; Bystroff *et al.*, 1990) are such that direct proton transfer from the carboxyl to N5 of FH_2 cannot occur. Among the possible routes which have been proposed are proton transfer via bound water molecules and transient protonation of O4 (Bolin *et al.*, 1982; Gready, 1985). An alternative is that the proton comes from the solvent and the carboxyl remains in the protonated state (Taira *et al.*, 1987; Uchimaru *et al.*, 1989; Bystroff *et al.*, 1990; Brown & Kraut, 1992).

We have earlier reported the preparation of the mutant of *L. casei* dhfr in which the aspartic acid at position 26 is replaced by asparagine (dhfr D26N[2]) and shown that this substitution abolishes the pH dependence of a conformational equilibrium in the enzyme−folate−$NADP^+$ complex (Jimenez *et al.*, 1989). We now describe the kinetic properties of this mutant; to understand these, it is important to know any structural consequences of the substitution, and we also describe a qualitative structural characterization of the mutant by NMR spectroscopy. The effect of this substitution on catalysis is found to be much less than that of the corresponding substitution of the *E. coli* enzyme, and the possible roles of Asp26 in catalysis are discussed in the light of this observation.

MATERIALS AND METHODS

Substrates, Coenzymes, and Inhibitors. Folic acid, 7,8-dihydrofolate (FH_2), 5,6,7,8-tetrahydrofolate (FH_4), methotrexate, and trimethoprim (Sigma Chemical Co.) were of the highest purity commercially available and used without further purification. Concentrations were determined from absorbance measurements using the following extinction coefficients: folic acid, 27 600 M^{-1} cm^{-1} at 282 nm, pH 7.0 (Rabinowitz, 1960); FH_2, 28 000 M^{-1} cm^{-1} at 282 nm, pH 7.4 (Dawson *et al.*, 1969); FH_4, 28 000 M^{-1} cm^{-1} at 297 nm, pH 7.5 (Kallen & Jencks, 1966); and methotrexate, 22 100 M^{-1} cm^{-1} at 302 nm, in 0.1 M KOH (Seeger *et al.*, 1949).

$NADP^+$ and NADPH were obtained from Sigma Chemical Co. NADPH was purified prior to use by anion exchange (Orr & Blanchard, 1984), using FPLC with a Pharmacia Mono Q column, to remove a breakdown product that is a potent inhibitor of dhfr activity at low pH. The deuterated coenzyme NADPD [(4*R*)-[^{2}H]NADPH] was prepared by reducing $NADP^+$ using alcohol dehydrogenase in the presence of [$^2H_6$]ethanol (Stone & Morrison, 1982) and purified in the same way. NADPH concentration was determined using an extinction coefficient of 6200 M^{-1} cm^{-1} at 340 nm, pH 7.5.

Protein Expression and Purification. The gene for the mutant of *L. casei* dhfr in which Asp26 is replaced by Asn was constructed by oligonucleotide-directed mutagenesis (Jimenez *et al.*, 1989). Both wild-type and mutant proteins were expressed in *E. coli* using a pMAC5-14 vector (Andrews *et al.*, 1991) in which expression is under the control of a temperature-sensitive λ repressor. Cells were grown on a 12 L scale in an LH Fermenter and induction was at 40 °C for 6 h. The enzyme was purified as described by Dann *et al.* (1976). Protein purity was checked by running native, SDS, and isoelectric focusing polyacrylamide gels. Enzyme concentrations were determined by absorbance spectroscopy (using ϵ_{280} = 30 500 M^{-1} cm^{-1}; Andrews *et al.*, 1989) and fluorescence titration with methotrexate (Dann *et al.*, 1976).

Equilibrium Binding Constants. The equilibrium binding constants of substrates, inhibitors, and coenzyme dhfr and to dhfr D26N were measured fluorimetrically using the methods previously described (Dunn *et al.*, 1978; Birdsall *et al.*, 1980). Ligand binding was measured by following the quenching of protein fluorescence using a Perkin-Elmer LS-5 luminescence spectrometer. Experiments were carried out at 25 °C using a buffer containing 50 mM potassium phosphate, pH 6.5, 500 mM KCl, 1% lactose. To correct for absorption of the excitation light by the ligand, a tryptophan solution was titrated under the same conditions as used for the protein titration (Birdsall *et al.*, 1983).

Steady-State Kinetics. All measurements over the pH range 5−9 were performed at 25 °C in KMB buffer (25 mM tris(hydroxymethyl)aminomethane, 25 mM ethanolamine, 50 mM 4-morpholinoethanesulfonic acid, 500 mM KCl). The ionic strength of this buffer remains constant over this pH range (Ellis & Morrison, 1982). Measurements between pH 4.5 and 5 were performed using 15 mM sodium acetate/500 mM KCl buffer.

Enzyme and NADPH were pre-equilibrated at 25 °C, and the reaction was initiated by the addition of FH_2. Initial velocities were measured by monitoring the change in absorbance at 340 nm that accompanies the reaction ($\Delta\epsilon$ = 11 800 M^{-1} cm^{-1}; Stone & Morrison, 1982). For k_{cat} measurements, substrate concentrations of 50 μM NADPH and 50 μM FH_2 were used. At low pH, the coenzyme is oxidized at a measurable rate in the absence of enzyme, and

[2] The nomenclature used is that commonly employed, in which the mutant enzyme is named by using the one-letter code for amino acids; thus dhfr in which aspartic acid 26 has been replaced by asparagine is denoted dhfr D26N. Dhfr without qualification refers to the wild-type enzyme. Unless specifically stated, the *L. casei* enzyme is referred to in all cases.

this rate was measured and subtracted from that of the enzyme-catalyzed reaction. Measurements of folate reduction were carried out in a similar way; the concentration of folate was 100−300 μM, and the $\Delta\epsilon$ value used was 18 900 M^{-1} cm^{-1} (Matthews & Huennekens, 1963). The k_{cat} values for NADPH and NADPD as the coenzyme were determined for the pH range 5−8.5, and the kinetic isotope effect [$k_{cat(NADPH)}/k_{cat(NADPD)}$] was calculated for each pH value.

Pre-Steady-State Kinetics. Pre-steady-state kinetic measurements were made using an Applied Photophysics (model SF.17MV) stopped-flow spectrometer operating in either a fluorescence or an absorbance mode, with a 1 mm sample cell. At least four runs were averaged for each set of solution conditions, and the data was analyzed by using an iterative nonlinear regression package supplied by Applied Photophysics.

The rate constant for hydride ion transfer from NADPH to FH_2 was determined from the initial rate of product formation after rapidly mixing the enzyme−NADPH complex with FH_2. Dhfr or dhfr D26N (1 μM) was preincubated with NADPH (20 μM); the reaction was initiated by mixing with an equal volume of FH_2 (50 μM) and monitored by measuring the decrease in absorbance at 340 nm.

The rate constant for the dissociation of FH_4 from the enzyme−NADPH−FH_4 ternary complex was measured by competition experiments using fluorescence detection (excitation at 290 nm, emission measured using a 350 nm interference filter). The enzyme concentration was 1 μM, with 5 μM NADPH, 5 μM FH_4, and 20 μM methotrexate as the competing ligand. The observed rate of fluorescence change, k_{obs}, was confirmed to be equal to the dissociation rate constant for FH_4 by showing that k_{obs} was independent of the concentration of methotrexate.

NMR Spectroscopy. The binary methotrexate complexes of the wild-type and mutant enzymes were obtained by eluting the enzyme from the affinity column with 2 mL of 5 mM methotrexate, pH 8.5. The eluent was dialyzed against 50 mM phosphate, 200 mM KCl, pH 6.5, to change the buffer and remove excess methotrexate. The volume was reduced to 0.4 mL and 0.05 mL of ^2H_2O added to give a final enzyme concentration of ~5 mM for dhfr and 1−2 mM for dhfr D26N.

^{1}H NMR spectra were recorded at 600 MHz on a Bruker AMX-600 spectrometer at 308 K. Typical parameters included 512 t_1 points, 4096 points in t_2, a spectral width of 8475 Hz, and a relaxation delay of 1.2 s. Data sets were zero-filled to 2048 in t_1 and multiplied by a phase-shifted sine-bell-squared function in both dimensions prior to transformation. Chemical shift values are reported relative to DSS. Two-dimensional NOESY (100 ms mixing time) and DQF-COSY spectra were recorded using the methods of Bodenhausen *et al.* (1984) and Rance *et al.* (1983), respectively. In both experiments a 60 ms SCUBA sequence was inserted immediately after the presaturation period and, in the case of NOESY, during the mixing time (Brown *et al.*, 1988). When the labile protons were to be observed, a jump-return observe pulse (Plateau & Gueron, 1982) was incorporated into the standard sequences; these spectra were recorded at 280 K using a spectral width of 18 519 Hz. A delay of 33 μs was used for the jump-return sequence to optimize detectability of signals at ~17 ppm. Cross-peak intensities were determined by nonlinear curve fitting in two dimensions using the program NDVOL (Sze *et al.*, 1995).

RESULTS AND DISCUSSION

Purity of the Mutant Enzyme. In the heterologous expression system used to produce the Asp26 → Asn mutant of *L. casei* dhfr (dhfr D26N), the host (*E. coli*) dhfr is readily and completely separated from *L. casei* dhfr at the anion exchange step of the purification procedure, facilitating the preparation of pure mutant enzyme. The purified dhfr D26N gave a single band on native, SDS, and isoelectric focusing polyacrylamide gels. The single charge difference between the mutant and wild-type enzymes leads to a change in the isoelectric point from 6.25 (±0.1) in the wild-type [see also Dann *et al.* (1976)] to 6.9 (±0.1) in the mutant, and isoelectric focusing thus affords a simple and powerful method for detecting any deamidation of dhfr D26N to wild-type enzyme. No such deamidation was detected during the work reported here [in agreement with the results obtained by Howell *et al.* (1986) with the corresponding mutant of *E. coli* dhfr].

Structural Comparisons: Methotrexate Binding. In order to identify any structural consequences of the substitution of Asp26, we have compared the ultraviolet and ^{1}H NMR spectra of the methotrexate complexes of the wild-type and mutant enzymes. This complex was chosen since its ^{1}H NMR spectrum has been almost completely assigned for the wild-type enzyme (Birdsall *et al.*, 1990; Carr *et al.*, 1991; Soteriou *et al.*, 1993), enabling a detailed comparison to be made.

Charge State of Bound Methotrexate. It is known from ^{1}H NMR and ultraviolet difference spectroscopy that when methotrexate or trimethoprim are bound to dhfr they are protonated on N1 (Hood & Roberts, 1978; Cocco *et al.*, 1981; Roberts *et al.*, 1981; Birdsall *et al.*, 1989). This implies a considerable increase in the pK_a value for this protonation on binding which, from the crystal structure, is due to the formation of an ion pair between the protonated pteridine and the Asp 26 carboxylate (Bolin *et al.*, 1982). The binding of the inhibitor methotrexate to both the wild-type and mutant enzymes was too tight to be measured accurately by fluorescence quenching. We therefore examined the binding of the α,γ-diamide analogue of methotrexate, which binds less tightly (Antonjuk *et al.*, 1984). As can be seen from Table 4, the binding of this methotrexate analogue is only 4-fold weaker to dhfr D26N than to the wild-type enzyme at pH 6.5. Given the apparent importance of the ion pair between the pteridine and Asp26 in the binding of methotrexate and related inhibitors, it is at first glance surprising that the asparagine substitution has such a small effect on the binding of methotrexate α,γ-diamide. However, this is not a simple comparison.

Ultraviolet difference spectroscopy (data not shown[3]) demonstrated that, while the difference spectrum for binding to dhfr bears a clear resemblance to that between neutral and protonated methotrexate (Hood & Roberts, 1978), there is no such resemblance for the difference spectrum generated on binding to dhfr D26N. This indicates that methotrexate is not protonated on N1 when bound to dhfr D26N. A similar conclusion was drawn from the ultraviolet difference

[3] The ultraviolet difference spectrum generated on methotrexate binding to *L. casei* dhfr D26N is closely similar to that reported for the *E. coli* dhfr D27N mutant (Howell *et al.*, 1986).

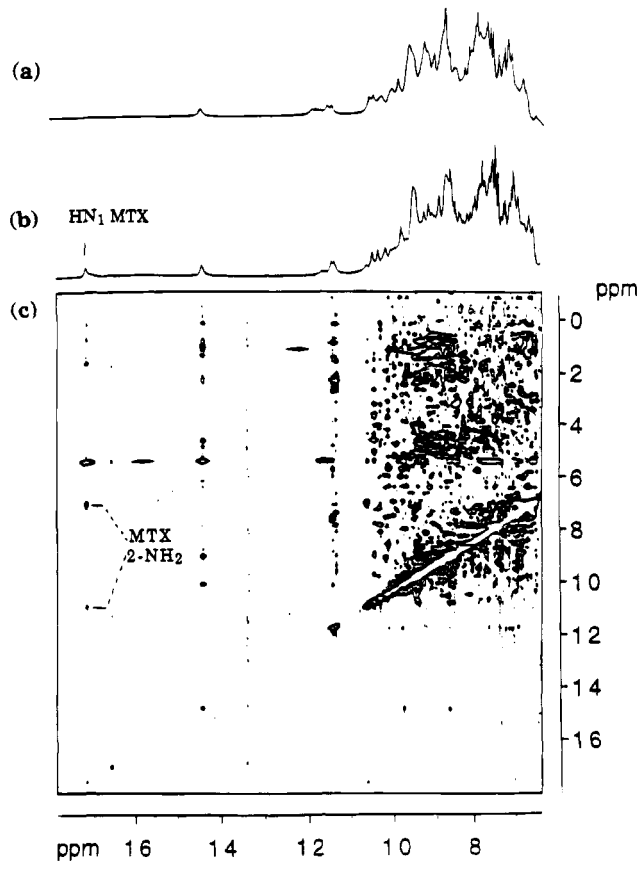

FIGURE 1: Low-field region of the ^1H NMR spectra of the methotrexate complexes of (a) dhfr D26N and (b) dhfr in H_2O. (c) Section of a NOESY spectrum showing the cross-peaks used to assign the resonance at 17 ppm in panel b to the N1 proton of bound methotrexate.

spectra generated on methotrexate binding to *E. coli* dhfr D27N (Howell *et al.*, 1986).

Further evidence that methotrexate is not protonated in its complex with dhfr D26N was obtained from ^1H NMR. In the spectrum of the dhfr-methotrexate complex in H_2O, the N1 proton resonance is observed at very low field, 17 ppm (Figure 1b). The assignment of this resonance is based on a NOESY experiment (Figure 1c), which shows key NOEs to the 2-amino protons as well as to the protons of a number of neighboring amino-acid residues. In the spectrum of the dhfr D26N–methotrexate complex, this low-field resonance is absent (Figure 1a), consistent with the conclusion that in this complex the ligand is not protonated on N1.

The modest, 4-fold, effect of the mutation on the binding of methotrexate α,γ-diamide (which is similar in magnitude to the 27-fold decrease in affinity for methotrexate reported for the *E. coli* D27N mutant; Howell *et al.*, 1986) cannot therefore be used to assess the contribution of the ion pair with Asp26 to binding, since methotrexate is in the *neutral* (high-pH) state in the complex, but the asparagine residue "mimics" the *protonated* (low-pH) form of aspartic acid.

Changes in the Structure of the Complex. Illustrative comparisons of the NMR spectra of dhfr and dhfr D26N are shown in Figures 2 and 3, from which it is clear that the substitution has very limited effects on the spectrum of the enzyme–methotrexate complex, indicating that there is very little overall change in the protein structure. The observed chemical shift differences of protein resonances of greater than 0.10 ppm are summarized in Table 1; these involve only 13 residues. The most pronounced effects on chemical shift are on the protons of Trp 21; crystallographic data has shown the indole proton of this residue to be an integral part of a conserved hydrogen-bonding network involving the inhibitor, Asp 26, and a bound water molecule (Bolin *et al.*, 1982; Figure 4).

The chemical shifts of the protons of the pteridine ring of free and bound methotrexate (except the N1 proton, which has been discussed above) are summarized in Table 2. The individual protons of the 2- and 4-amino groups have not been separately assigned in free methotrexate (indeed only one average signal is seen for the 4-amino protons), so that exact values for the change in chemical shift of these protons on binding cannot be calculated. We can, however, compare the shift difference between neutral and protonated free methotrexate with that between methotrexate bound to wild-type and mutant dhfrs. For the 4-amino protons, the shift difference has the same sign in both cases, although the difference between the enzyme complexes is greater than that between the two charge states of the free ligand. For

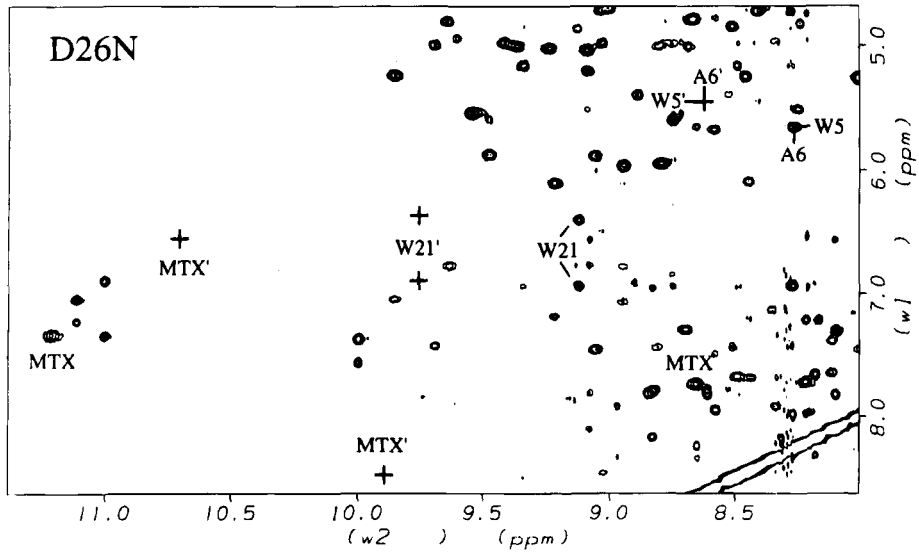

FIGURE 2: Part of the NOESY spectrum of the methotrexate complex of dhfr D26N. Crosses and primed residue names indicate the position in the spectrum of the complex of the wild-type enzyme of cross-peaks which differ notably in position between the mutant and wild-type enzymes.

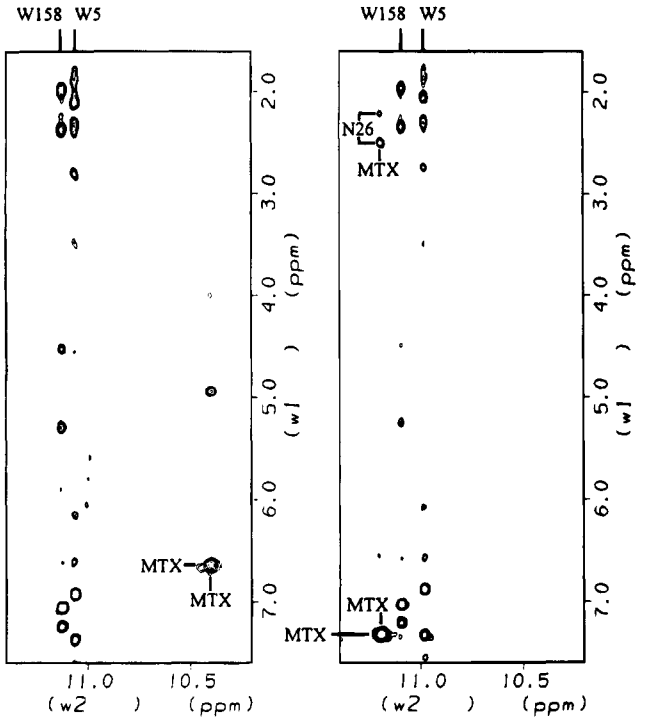

FIGURE 3: Low-field region of the NOESY spectra of the methotrexate complexes of (a) dhfr and (b) dhfr D26N. Cross-peaks between a 2-amino proton of methotrexate and the β-protons of Asn26 are highlighted in the spectrum of the mutant complex.

Table 1: ^{1}H NMR Chemical Shift Differences between Wild-Type dhfr and the D26N Mutant[a]

residue		δ dhfr[b]	δ D26N[c]	$\Delta\delta$[d]
Leu4	$C_\gamma H$	2.31	2.43	0.12
Trp5	$C_\alpha H$	5.51	5.65	0.14
Ala6	NH	8.57	8.27	−0.30
Leu12	$C_\alpha H$	4.35	4.47	0.12
Ile13	NH	8.94	8.81	−0.13
Lys15	$C_\beta H_1$	1.68	1.56	−0.12
	$C_\delta H_1$	1.67	1.57	−0.10
Asp16	NH	10.13	9.97	−0.16
Trp21	$C_\beta H_1$	2.87	2.43	−0.44
	$C_\beta H_2$	2.48	2.37	−0.11
	$C_{\epsilon 1} H$	9.69	9.12	−0.57
	$C_{\epsilon 3} H$	6.00	6.12	0.12
Leu23	$C_\delta H_3$	0.04	0.22	0.18
Tyr29	$C_\delta H$	7.92	7.80	−0.12
Phe30	$C_\delta H$	6.71	6.57	−0.14
Leu118	$C_\delta H_3$	−0.55	−0.39	0.16
Lys127	NH	8.76	8.64	−0.12

[a] All assigned proton resonances for which the chemical shift difference ($\Delta\delta$) is greater than ±0.10 ppm are included. [b] Chemical shift (ppm) in the methotrexate complex of wild-type dhfr. [c] Chemical shift (ppm) in the methotrexate complex of dhfr D26N. [d] Chemical shift difference between wild-type and mutant enzymes; positive values indicate the resonance is further downfield in the mutant.

the 2-amino protons, on the other hand, the signals in the complex with dhfr D26N are downfield of those in the wild-type complex, the opposite direction to that expected on the basis of the difference in charge state alone. This amino group, of course, is involved in a hydrogen-bonding interaction to Asp26 in the wild-type enzyme and probably to Asn26 in the mutant (Figure 4), and thus its environment differs significantly in the two complexes.

All the protons which show chemical shift differences between the wild-type and mutant enzymes are within 8 Å of residue 26 in the crystal structure of the enzyme−methotrexate−NADPH complex. On this basis, we made a

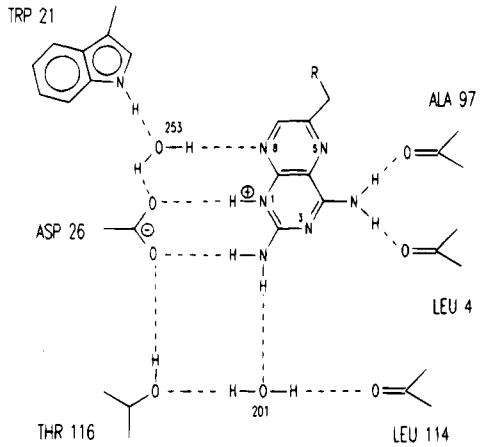

FIGURE 4: Environment of Asp26 in the structure of the *L. casei* dhfr−methotrexate−NADPH complex based on the data of Bolin *et al.* (1982).

quantitative comparison of the intensities of the NOE cross-peaks (see Figure 3) of residues within an 8 Å sphere centered on the site of mutation. In order to calculate the scaling factor between the intensities in the two NOESY spectra and to estimate the precision of the measurements, we compared the intensities of the NOE cross-peaks between neighboring protons on the aromatic rings, where the interproton distance is constant. The correlation between the intensities of these reference cross-peaks in the spectra of dhfr and dhfr D26N is shown in Figure 5a, in which the intensities are plotted as their sixth roots to allow an estimation of the deviations in terms of interproton distances. After scaling, all the deviations for the reference cross-peaks are well within a ±10% distance variation. A total of 132 cross-peaks from residues in the 8 Å sphere around residue 26 could be unambiguously assigned in both the dhfr and dhfr D26N spectra, and a comparison of their intensities is presented in Figure 5b. The deviation is clearly greater than for the reference cross-peaks, indicating that there are real intensity, and hence interproton distance, differences. These differences are all within ±20% in terms of distance, corresponding to an upper limit of 0.5−1 Å to the difference in interproton distances.

The only *new* cross-peaks to appear in the spectrum of the mutant which could be unambiguously assigned were due to cross-relaxation between a 2-amino proton of methotrexate and the β-protons of Asn26. The corresponding cross-peak to the side chain of Asp26 in the wild-type was not observed (see Figure 3). In the crystal structure of the enzyme−NADPH−methotrexate complex, the distance between these protons is ~5 Å, close to the threshold value for observation of NOEs under these experimental conditions, and only a modest decrease in the interproton distance in dhfr D26N would be required to generate an observable cross-peak. The other NOE cross-peaks whose intensities have changed by a least a factor of 2 are listed in Table 3.

Combining the information from chemical shift and NOE changes, we can identify regions of the structure which differ between the wild-type and mutant enzymes:

(a) Around the 2-amino group of methotrexate. In addition to the new NOE between a 2-amino proton and Asn26 itself noted above, there is a decrease in intensity of the NOE cross-peak between the 2-amino group and Ala6. The NH of Ala6 shows a significant change in chemical shift, and there are large changes in the shift of the protons of the 2-amino group itself.

Table 2: ^{1}H NMR Chemical Shifts of the Pteridine Protons of Methotrexate, Free and in Its Binary Complexes with Wild-Type dhfr and the D26N Mutant

	δ			δ		
proton	neutral MTX	protonated MTX	Δδ, neutral − protonated	dhfr−MTX	dhfr D26N−MTX	Δδ, dhfr D26N − dhfr
2-NH_2	7.60	8.10	−0.50	10.40	11.21	0.81
	7.34	7.90	−0.56	6.64	7.34	0.70
4-NH_2	6.30	6.96	−0.66	9.89	8.65	−1.24
				8.55	7.74	−0.81
C7-H	8.57	8.61	−0.04	7.86	7.74	−0.12

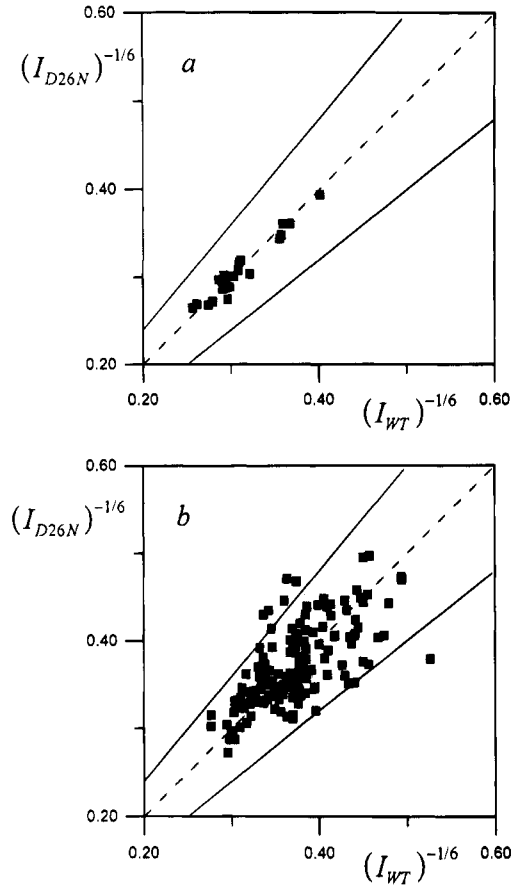

FIGURE 5: Comparisons of the intensities of selected NOE cross-peaks (measured using NDVOL; Sze *et al.*, 1995) in the spectra of the methotrexate complexes of dhfr and dhfr D26N. The sixth root of the intensity is plotted, so that the comparisons are approximately comparisons of interproton distances. (a) Comparison of reference cross-peaks, corresponding to intraresidue interactions between neighboring protons on aromatic rings. (b) Comparison of 132 NOE cross-peaks involving residues within 8 Å of Asp26. The dashed lines correspond to perfect agreement and the solid lines to differences of ±10%.

(b) Around the 4-amino group of methotrexate. There are changes in chemical shift and NOEs of protons of Leu4, whose carbonyl group hydrogen-bonds to the 4-amino group, and its neighbors Trp5 and Phe30, as well as changes in shift of the 4-amino protons.

(c) Trp21 and the loop between βA and αB. The chemical shift changes of the protons of Trp21 have already been noted, and a number of other residues in the βA−αB loop show modest but significant differences in chemical shift between the methotrexate complexes of dhfr and dhfr D26N; the exact conformation of this flexible loop appears to differ in different complexes of both *L. casei* and *E. coli* dhfr, and it has been proposed to play a role in ligand binding and catalysis [*e.g.*, Bystroff *et al.* (1990) and Li *et al.* (1992)].

Table 3: Assigned NOEs Differing by a Factor of 2 or More in Intensity between the Methotrexate Complexes of Wild-Type dhfr and the D26N Mutant

residue	proton	residue	proton	intensity ratio, D26N/wt[a]
Leu4	$C_{\delta 1}H_3$	Leu4	$C_\alpha H$	0.6
Leu4	$C_{\delta 1}H_3$	Trp5	NH	0.7
Leu4	$C_{\delta 2}H_3$	Phe30	$C_\delta H$	0.5
Trp5	$C_\alpha H$	MTX[b]	C4-NH_2	2.3
Trp5	NH	Val115	$C_{\gamma 2}H_3$	0.5
Ala6	$C_\beta H_3$	MTX[b]	C2-NH_2	0.5
Asp8	$C_{\beta 1}H$	Phe122	$C_\delta H$	2.9
Leu19	$C_{\delta 2}H_3$	MTX[b]	N1-H	0.3
Leu23	$C_{\delta 2}H_3$	Leu118	$C_{\delta 1}H_3$	0.3
Leu23	$C_{\delta 2}H_3$	Leu151	$C_{\delta 2}H_3$	0.5
Pro24	$C_{\beta 1}H$	His28	$C_{\epsilon 1}H$	0.3
Tyr29	$C_\delta H$	Phe30	NH	0.4
Phe30	$C_\delta H$	Arg31	$C_\alpha H$	2.1
Phe30	$C_\epsilon H$	Thr34	$C_\gamma H_3$	0.25
Leu118	$C_{\delta 2}H_3$	Phe122	$C_\epsilon H$	0.5
Leu118	$C_{\delta 2}H_3$	Phe122	$C_\xi H$	2.9
Phe122	$C_\delta H$	Phe122	$C_\alpha H$	3.5
Val144	$C_{\gamma 2}H_3$	Tyr155	$C_\delta H$	0.6
Tyr155	$C_\alpha H$	Tyr155	$C_\delta H$	2.6

[a] Ratio of the cross-peak intensity in the spectrum of the dhfr D26N−methotrexate complex to that in the spectrum of the dhfr−methotrexate complex. [b] Methotrexate.

There are changes in NOEs involving Leu19 in this loop and Leu118, a close neighbor of Trp 21.

It is thus clear that the substitution of Asp26 by Asn has only very local effects on the structure of the enzyme−methotrexate complex of *L. casei* dhfr, as has been shown crystallographically for this complex of the corresponding D27N mutant of the *E. coli* enzyme (Howell *et al.*, 1986). The only crystal structure available for *L. casei* dhfr is that of the ternary enzyme−methotrexate−NADPH complex (Bolin *et al.*, 1982), so that a detailed analysis of the structural effects of the substitution in the binary complex must await completion of calculations of the solution structure of this complex which are in progress (I. L. Barsukov, K.-H. Sze, M. G. Casarotto, B. Birdsall, V. I. Polshakov, J. Feeney, and G. C. K. Roberts, unpublished work). Some qualitative effects are, however, clear. The fact that methotrexate is unprotonated in the complex with the mutant implies that it is likely to be the NH_2 of the amide group of Asn26 which hydrogen-bonds to N1 of methotrexate [see also Howell *et al.* (1986)]; this in turn would imply a reorientation of the water molecule which lies between residue 26 and Trp21 (see Figure 4). This may result in breaking the hydrogen bond between this water and the indole NH of Trp21 and a slight reorientation of the indole ring. At the same time, the changes around the 2- and 4-amino groups of methotrexate imply a slight reorientation of the pteridine ring of the bound inhibitor; the changed charge state of this ring will result in changes in electron distribution and perhaps in the relative strengths of the

Table 4: Equilibrium Dissociation Constants for Ligands Binding to Wild-Type dhfr and the D26N Mutant

ligand	K_d (μM) dhfr	K_d (μM) dhfr D26N
FH_2	0.24 (±0.02)	1.6 (±0.06)
folate	7.8 (±0.3)	10.0 (±0.8)
NADPH	0.01 (±0.001)	0.01 (±0.001)
methotrexate α,γ-diamide	0.15 (±0.004)	0.6 (±0.06)

Table 5: Steady-State Kinetic Parameters of Wild-Type dhfr and the D26N Mutant

kinetic parameter	dhfr pH 5.5	dhfr pH 7.5	dhfr D26N pH 5.5	dhfr D26N pH 7.5
		FH_2		
K_M (μM)	1.0 (±0.5)	1.0 (±0.5)	1.6 (±0.3)	1.4 (±0.2)
k_{cat} (s^{-1})	25 (±0.8)	10 (±0.5)	5.5 (±0.1)	1.1 (±0.02)
k_{cat}/K_M ($M^{-1}\,s^{-1}$)	2.5×10^7	1.0×10^7	3.4×10^6	7.9×10^5
		Folate		
K_M (μM)	0.6 (±0.1)	29 (±4)	0.6 (±0.1)	16 (±2)
k_{cat} (s^{-1})	nd[a]	0.06 (±0.003)	nd	0.0024 (±0.0001)
k_{cat}/K_M ($M^{-1}\,s^{-1}$)	nd	2×10^3	nd	1.5×10^2

[a] *nd*: not determined.

hydrogen bonds involving these amino groups. In the crystallographic study of the *E. coli* mutant, an upper limit to atomic movements of 0.2 Å was estimated (Howell *et al.*, 1986). Given the great sensitivity of NOEs to changes in interproton distances, there is no reason, pending the results of the detailed calculations, to suggest that the local structural changes in the *L. casei* enzyme are much greater than this.

Ligand Binding. The binding of substrates, inhibitors, and coenzyme to dhfr can readily be measured by following the quenching of the intrinsic fluorescence of the protein which accompanies ligand binding (Birdsall *et al.*, 1980). Equilibrium dissociation constants for substrates, coenzyme, and inhibitor binding to dhfr and dhfr D26N determined in this way are given in Table 4. The substitution has no effect on the binding of the coenzyme NADPH and the poor substrate folate and only a small, 7-fold, effect on the binding of the better substrate FH_2.

Kinetics of Reduction of FH_2. Steady-State Kinetics. The steady-state kinetic parameters for FH_2 reduction by dhfr and dhfr D26N are summarized in Table 5. It is apparent that the effects of replacing the active-site carboxylate by a carboxamide are relatively modest. There is only a minimal effect on the K_M for FH_2, in line with the small increase in K_d (Table 4). The values of k_{cat} are decreased by no more than a factor of 9 and those of k_{cat}/K_M by no more than a factor of 13. This is in marked contrast to the effects of the corresponding substitution (D27N) in *E. coli* dhfr, which leads to decreases of a factor of 300 in k_{cat} and a factor of 1.1×10^4 in k_{cat}/K_M for FH_2 at pH 7 (Howell *et al.*, 1986).

The pH dependence of k_{cat} with FH_2 as substrate for dhfr and dhfr D26N is shown in Figure 6a. The decrease in k_{cat} with increasing pH for the wild-type enzyme can be fitted to a single apparent pK_a of 7.3 (Andrews *et al.*, 1989). There is a change in the rate-limiting step with pH, from product (FH_4) release at low pH to hydride ion transfer at high pH (Andrews *et al.*, 1989), as reflected in the pH dependence of the deuterium isotope effect on k_{cat} when NADPD is used

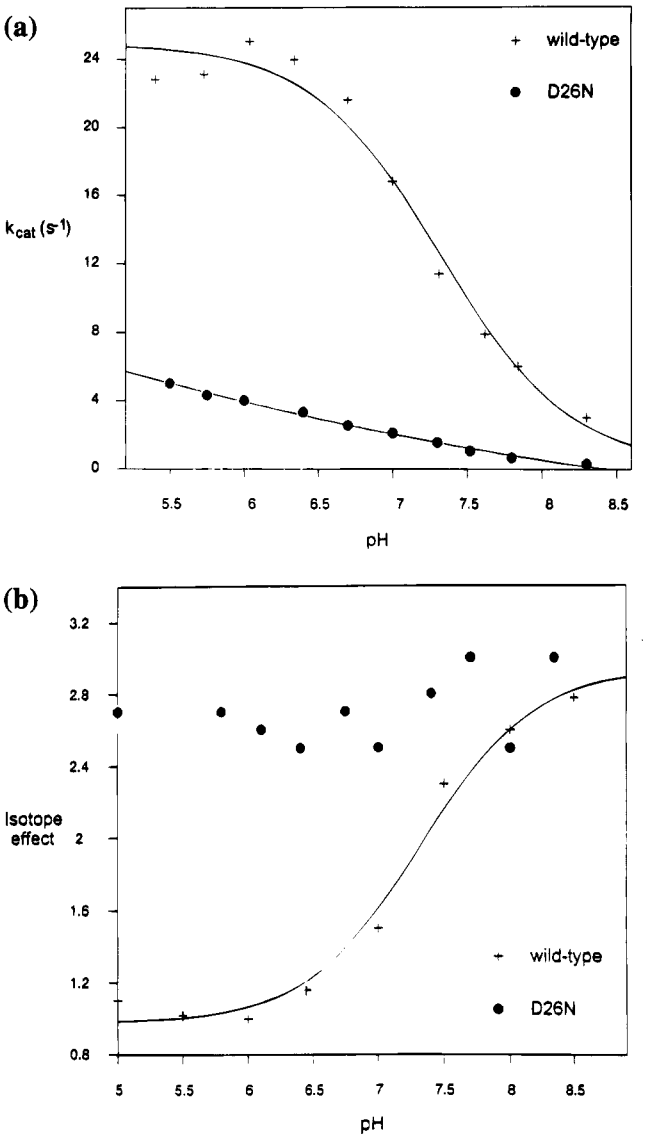

FIGURE 6: (a) pH dependence of k_{cat} for FH_2 reduction catalyzed by dhfr (crosses) and dhfr D26N (solid circles). (b) pH dependence of the deuterium isotope effect on k_{cat} [$k_{cat(NADPH)}/k_{cat(NADPD)}$] for FH_2 reduction catalyzed by dhfr (crosses) and by dhfr D26N (solid circles).

as coenzyme (Figure 6b). The true pK of the group which must be protonated for catalysis to occur (Stone & Morrison, 1984) has been determined to be 6.0 (Andrews *et al.*, 1989), and as noted above, it is widely assumed that this group is Asp26.

The pH dependence of k_{cat} for dhfr D26N is quite different from that of the wild-type enzyme, showing simply a gradual, almost linear, increase in k_{cat} with decreasing pH (Figure 6a). This is very similar to the behavior reported for *E. coli* dhfr D27N (Howell *et al.*, 1986) and is consistent with, though it does not prove, the suggestion that Asp26 is indeed the group with a pK of 6.0 which determines the pH dependence of k_{cat}. In the case of the *E. coli* dhfr D27N mutant, Howell *et al.* [1986; see also Warren *et al.* (1991)] proposed that the pH dependence of k_{cat} could be accounted for by a model involving binding of FH_2 protonated on N5 (pK_a = 2.9; Maharaj *et al.*, 1990). This model does not fit the data for *L. casei* dhfr D26N well, since it predicts a much steeper increase in k_{cat} with decreasing pH than is actually observed; a similar discrepancy exists for the *E. coli* mutant (Howell *et al.*, 1986; Warren *et al.*, 1991).

Table 6: Rate Constants for Dissociation of FH_4 from the Enzyme−FH_4−NADPH Complexes of Wild-Type dhfr and the D26N Mutant

	dissociation rate constant (s^{-1})	
pH	dhfr	dhfr D26N
5.5	29 (±0.8)	112 (±2)
6.0	44 (±1)	174 (±5)
7.0	97 (±4)	162 (±4)
8.0	76 (±2)	121 (±2)
9.0	73 (±2)	132 (±2)

There is also a marked difference in the pH dependence of the kinetic deuterium isotope effect, which for dhfr D26N is approximately 2.7 across the whole pH range from 5.0 to 8.0 (Figure 6b). This indicates that the rate-limiting step for catalysis in dhfr D26N differs from that in the wild-type enzyme, with hydride ion transfer being rate-limiting from pH 5 to 8.

Pre-Steady-State Kinetics. The two contributions to the rate-limiting step in catalysis, hydride ion transfer and FH_4 release, can be measured individually by stopped-flow methods. The rate of hydride ion transfer (k_{hyd}) in the wild-type enzyme has been measured from the "burst" of product formation which is equivalent to the first turnover of the enzyme (Andrews *et al.*, 1989). Equivalent experiments with dhfr D26N at several pH values, under conditions where the first turnover could readily be measured, showed no evidence for a "burst" of product formation; rather a linear rate was observed. This provides confirmation that the hydride ion transfer is the rate-limiting step in catalysis by this mutant.

The kinetically important pathway for FH_4 release involves dissociation of $NADP^+$ from the enzyme−FH_4−$NADP^+$ complex followed by binding of NADPH and then dissociation of FH_4; due to negative cooperativity between FH_4 and NADPH, dissociation of FH_4 is fastest from the enzyme−FH_4−NADPH complex (Andrews *et al.*, 1989). The rate constants for dissociation of FH_4 from the enzyme−FH_4−NADPH complex for dhfr and dhfr D26N are shown in Table 6. Across the pH range studied, product release is 1.6−4-fold faster in the mutant than in the wild-type enzyme and much faster than k_{cat}; for example, at pH 5.5, k_{cat} is 5.5 s^{-1} and the rate of product release is 112 s^{-1}.

Thus, the combination of the deuterium isotope effect and the pre-steady-state kinetic measurements demonstrates clearly that, across the whole pH range 5−8, the rate-limiting step for FH_2 reduction by dhfr D26N is hydride ion transfer, and the rate of hydride transfer is thus equal to the steady-state k_{cat}. In order to make a meaningful comparison between the wild-type and mutant enzyme, we must compare like with like that is, we must compare the rates of hydride ion transfer, not simply the k_{cat} values. At pH 5.5, the rates of hydride ion transfer are 340 and 5.5 s^{-1} for dhfr and dhfr D26N, respectively, a 62-fold decrease due to the substitution (significantly greater than its effect on k_{cat}). At pH 7.5, the substitution leads to a decrease in hydride transfer rate by only a factor of 9.

Similar comparisons can be made for *E. coli* dhfr and the D27N mutant using estimates from published data. The values given by Farnum *et al.* (1991) were obtained by fitting the experimental data for the mutant as a function of pH to the model of Howell *et al.* (1986) and extrapolating to low pH (fully protonated FH_2). In view of the uncertainty, noted above, as to whether this model fits the data adequately, we have sought to make the comparison on the basis of data measured at individual pH values. At pH 5.5 the rate of hydride ion transfer is approximately 750 s^{-1} for the wild-type enzyme (Fierke *et al.*, 1987). At this pH, k_{cat} for the D27N mutant is 0.5 s^{-1}; a direct measure of k_{hyd} has not been reported, but the deuterium isotope effect on k_{cat} has been reported to be 2.2 (Howell *et al.*, 1986). The magnitude of this isotope effect, compared to that of 3.0 on the hydride transfer step (Fierke *et al.*, 1987), indicates that hydride ion transfer may be only partly rate-limiting for *E. coli* dhfr D27N at this pH and suggests roughly equal contributions of hydride ion transfer and product dissociation (1 s^{-1} each). In the wild-type enzyme, the rate of product dissociation is 12 s^{-1} (Fierke *et al.*, 1987), and this would imply a decrease, rather than the increase reported here for the *L. casei* enzyme, in the rate of product dissociation (there is no direct information on this rate for the D27N mutant). Taking this estimate of k_{hyd} = 1 s^{-1} for the *E. coli* mutant at pH 5.5, we conclude that the D27N substitution leads to a 750-fold decrease in k_{hyd}. At pH 7.0, the measured values of k_{cat} are 30 s^{-1} for the wild-type and 0.1 s^{-1} for the D27N mutant (Howell *et al.*, 1986); for product dissociation to contribute to this rate in the mutant, it would need to have decreased 1000-fold. Assuming $k_{cat} = k_{hyd}$ for the mutant at pH 7.0, and taking k_{hyd} for the wild-type enzyme from Fierke *et al.* (1987), we estimate that the D27N substitution leads to a 2000-fold decrease in k_{hyd} at pH 7.0. Thus, substitution of the active site aspartate residue in *E. coli* dhfr by asparagine decreases the rate of hydride ion transfer by about 1000-fold, in marked contrast to the 9−62-fold decrease seen for the *L. casei* enzyme.

Kinetics of Reduction of Folate. The steady-state kinetic parameters for folate reduction by dhfr and dhfr D26N are given in Table 5, and the pH dependence of k_{cat} is shown in Figure 7. Unlike that for FH_2, the K_M for folate is pH dependent, increasing almost 50-fold between pH 5.5 and 7.5, but as for FH_2, the D26N mutation has very little effect on K_M at either pH, in line with the lack of effect on K_d (Table 4). Folate is a much poorer substrate for the enzyme than FH_2 (k_{cat} is 700−800 times lower), but the magnitude of the effects of the D26N mutation on k_{cat} and k_{cat}/K_M are similar to those seen for FH_2. Measurements of the deuterium isotope effect with NADPD indicate that hydride ion transfer is rate-limiting across the pH range both for the wild-type enzyme (Andrews *et al.*, 1989) and for the mutant (data not shown), and the effect of the substitution on the rate of hydride ion transfer at pH 7.5 is therefore rather greater for folate reduction (25-fold) than for FH_2 reduction (9-fold). A quantitative comparison with the effects of the corresponding substitution in *E. coli* dhfr is not possible, since folate is a very poor substrate for the *E. coli* enzyme, and folate reduction by the D27N mutant was not detectable (Howell *et al.*, 1986).

The pH dependence of k_{cat} for folate reduction by the wild-type enzyme (Figure 7) is satisfactorily described by two pK_as, of 5 (±0.36) and 7.3 (±0.2), with k_{cat} being maximal when both groups are protonated. (In an earlier report, the pH dependence was characterized by a single pK of 6.9, on the basis of data which did not extend as far to low pH; Andrews *et al.*, 1989.) The k_{cat} profile of dhfr D26N, by contrast, can be fitted to a single pK_a of 6.9 (±0.1). This suggests the simple explanation that the group with a pK of 5 is Asp26. (This should be a true pK value for this residue, since hydride ion transfer is rate-limiting across the pH range.) The identity of the group with a pK of 6.9−7.3 is

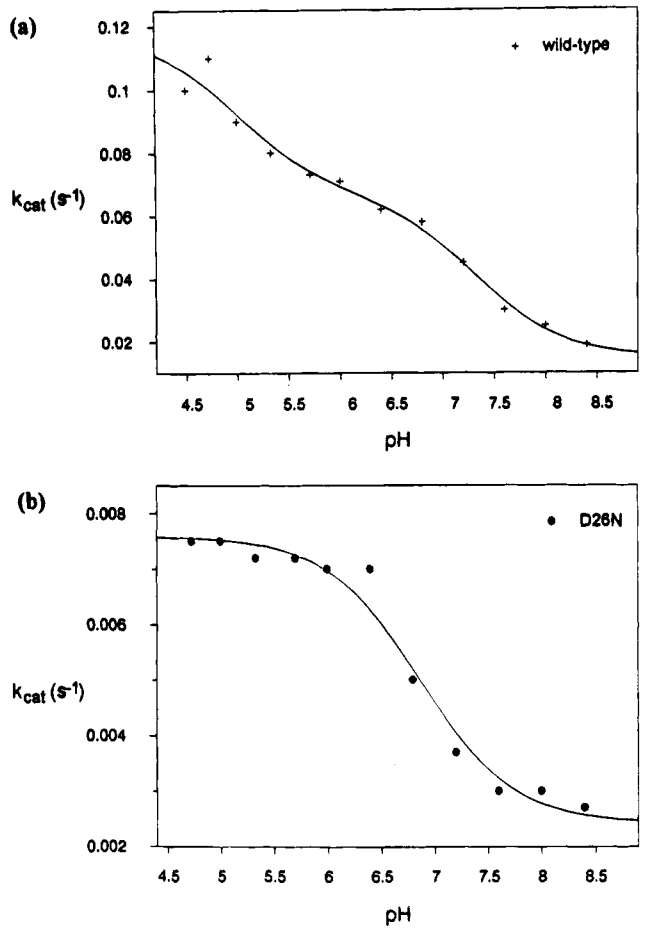

FIGURE 7: pH dependence of k_{cat} for folate reduction catalyzed by (a) dhfr and (b) dhfr D26N. The lines represent the best fit to two and one pKs, respectively, as discussed in the text.

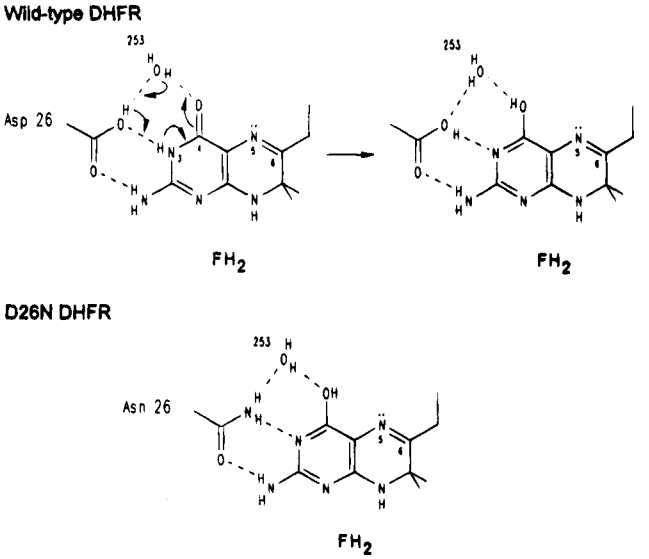

FIGURE 8: Possible roles of Asp26 and Asn26 in keto–enol tautomerism of FH_2.

less clear at present. An understanding of the pH dependence of reduction of folate is complicated by the existence of a pH dependent conformational equilibrium in the enzyme–folate and enzyme–folate–$NADP^+$ complexes, in which only one conformer has folate bound in the correct orientation for reduction to occur (Birdsall *et al.*, 1982, 1987, 1989b; Cheung *et al.*, 1993); it is not known whether a similar equilibrium exists in the catalytically functional enzyme–folate–NADPH complex. However, we have earlier shown that the pH dependence of this equilibrium is abolished in dhfr D26N (Jimenez *et al.*, 1989), so that the ionization with a pK ~ 7 cannot be associated with this conformational effect. As noted above, Asp26 is the only ionizable group on the protein which is close to the pteridine ring in the active site of the enzyme. However, the pK for deprotonation of N3 of the pteridine ring in the normal, keto, tautomer of free folate is 8.3 (Poe, 1977), raising the possibility that the pK ~ 7 which is seen in the pH dependence of k_{cat} corresponds to this ionization in bound folate; a second possibility is that it corresponds to the deprotonation of N8 (Davies *et al.*, 1990; see below). Cheung *et al.* (1993) have recently used ^{13}C NMR of labeled folate to study the tautomeric and ionization states of folate in the different conformational states of the enzyme–folate and enzyme–folate–$NADP^+$ complexes. They concluded that, in the conformation where folate is bound in the correct orientation for reduction, it is present as the keto tautomer, but up to pH 7.3 there were no changes in chemical shift of C2 or C4 which might indicate an ionization of N3. In the case of human dhfr, ^{15}N NMR studies (Blakley *et al.*, 1993) have recently shown that N3 remains protonated across the whole pH range in the enzyme–folate and enzyme–folate–$NADP^+$ complexes. Studies of a better model of the enzyme–folate–NADPH complex will be required to fully understand the origins of the pH dependence of folate reduction.

Role of Asp26 and Asn26 in Catalysis. The observation that in *L. casei* dhfr substitution of Asp26 by Asn leads to a decrease in the rate of hydride ion transfer from NADPH to FH_2 by only a factor of 62 at pH 5.5 and a factor of 9 at pH 7.5 does not suggest a uniquely essential role for this residue in catalysis. Comparable effects have been seen for substitutions of a number of other residues around the active site, such as the conserved tryptophan (W21 in *L. casei* dhfr; Thillet *et al.*, 1988; Andrews *et al.*, 1989; Warren *et al.*, 1991). In particular, this rather modest decrease is not readily compatible with mechanisms which involve an essential proton transfer from the carboxyl of Asp26 to bound water molecules and/or O4 of FH_2 and thence to N5 of the substrate.

However, a mechanism in which Asp26 remains in the protonated state throughout the catalytic cycle (Taira *et al.*, 1987; Uchimaru *et al.*, 1989; Bystroff *et al.*, 1990; Brown & Kraut, 1992) is more easily reconciled with the relatively high catalytic activity of the Asn26 enzyme, as illustrated in Figure 8. In this mechanism, the carboxyl of Asp26 and a crystallographically observed bound water molecule act to assist the transfer of a proton from N3 to O4 of FH_2, that is, the tautomerization from the keto to the enol form. The proton on O4 is then transferred, perhaps via another water molecule (Brown & Kraut, 1992), to N5; this would promote hydride ion transfer to C6 (Gready, 1985). The N5-protonated species could be either the keto or enol tautomer. In this scheme, Asp26 will assist the tautomerization of the bound substrate, but it is not clear to what extent it will act to favor the enol over the normal keto tautomer. It has been suggested that long-range electrostatic effects may have significant effects on the electron distribution in the pteridine ring (Bajorath *et al.*, 1991) and perhaps on the tautomeric equilibrium, but the calculation of these effects has been found to be critically dependent on the exact disposition of the water molecules around the active site (M. J. Sutcliffe, personal communication).

When the aspartic acid side chain is replaced by an asparagine, the same assistance in proton transfer in the keto−enol tautomerization is not possible, since an -NH_2 replaces an -ÖH. However, the asparagine can still serve to orient the substrate in essentially the same way as the aspartic acid. In particular, the Asn side chain can clearly interact more favorably with the enol than with the keto tautomer of the substrate. If the enolization of FH_2 is important for catalysis, then the Asp → Asn substitution would be, to this extent, a favorable one. On the other hand, only a small fraction of FH_2 exists in the enol form in free solution, and probably most important, the orientation of at least one of the crucial bound water molecules would have to be different in the mutant, and perhaps less favorable for proton transfer to N5 via O4. It may be that the balance between these factors determines the catalytic activity of the asparagine mutant. The marked difference in the effects of this substitution on the *L. casei* and *E. coli* enzymes, which are kinetically extremely similar (Andrews *et al.*, 1989), could then result from even small differences in the location and/or orientation of the bound water molecules in the substrate complex, or from differences in the position of the equilibrium between keto and enol tautomers of the bound substrate on the two enzymes.

Further insight into the mechanism will depend upon direct measurements of the charge state of Asp26 and the tautomeric and charge states of FH_2 in complexes as close as possible to the catalytically functional enzyme−FH_2−NADPH complex. Thus, we have recently prepared selectively [γ-^{13}C]Asp-labeled dhfr (Badii *et al.*, 1995), and NMR measurements of the pK of Asp26 in various complexes are in progress. An important step toward direct determination of the relevant charge states has recently been taken by Chen *et al.* (1994), who have reported difference Raman spectroscopy measurements of the pK of N5 of FH_2 when bound to *E. coli* dhfr. They report that this pK is less than 4.0 in the enzyme−FH_2 complex and the enzyme−FH_2−tetrahydroNADPH[4] complex but that N5 has a pK of 6.5 in the enzyme−FH_2−$NADP^+$ complex. This dramatic increase in pK is dependent on the presence of the active-site carboxyl, since it was not seen with the D27S mutant. Chen *et al.* (1994) propose that the group which determines the pH dependence of k_{cat} for FH_2 is not Asp27 (*E. coli* numbering) but N5 of the substrate,[5] the active-site carboxylate (unprotonated) acting to stabilize the protonated state of the dihydropteridine ring (Gready, 1985). However, the observation reported here that substitution of Asp26 with Asn has a relatively small effect on the rate of hydride transfer is hard to reconcile with an essential role for the negatively charged carboxylate, at least in the *L. casei* enzyme.

The mechanism of reduction of folate by dhfr remains still less well understood. Reduction of the 7,8-double bond should be promoted by protonation of N8 (Gready, 1985). A hydrogen bond to the backbone carbonyl of Ile5 (Bystroff *et al.*, 1990; *E. coli* numbering) might stabilize a protonated N8 of folate. Support for this comes from studies of the complex of human dhfr with 5-deazafolate, a tightly binding inhibitor which is protonated on N8 when bound to the enzyme (Davies *et al.*, 1990; Williams & Morrison, 1992), the protonated N8 of the inhibitor forming a hydrogen bond to the backbone carbonyl of Ile7 (Davies *et al.*, 1990; this residue is equivalent to Ile5 of *E. coli* dhfr). Davies *et al.* (1990) used the structure of this complex to construct a model of a proposed transition state for folate reduction by dhfr, in which N8 is protonated. However, it is not clear that the 5-deazafolate complex is a good model for the transition state (Williams & Morrison, 1992), and importantly, there is no obvious route by which a proton could be transferred to N8 from the solvent (Davies *et al.*, 1990). There is good NMR evidence that in the catalytically relevant conformation of the enzyme−folate and enzyme−folate−$NADP^+$ complexes, the substrate exists in the keto tautomer and is not protonated on N5 (Selinsky *et al.*, 1990; Cheung *et al.*, 1993; Blakley *et al.*, 1993). In any case, it is not clear that either enolization or protonation on N5 would promote reduction of the 7,8-bond. It may be that the apparently strained interactions discussed by Bystroff *et al.* (1990) and Davies *et al.* (1990) on the basis of the *E. coli* dhfr−folate−$NADP^+$ and human dhfr−folate and dhfr−5-deazafolate complexes are the principal sources of the rate acceleration for this substrate.

[4] TetrahydroNADPH is 1,4,5,6-tetrahydronicotinamide adenine dinucleotide phosphate.

[5] The conclusion that the pK of N5 is elevated in the catalytically functional enzyme−FH_2−NADPH complex depends on the assumption that the enzyme−FH_2−$NADP^+$ complex is a better model of the active complex than is the enzyme−FH_2−tetrahydroNADPH complex.

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REFERENCES

Andrews, J., Fierke, C. A., Birdsall, B., Ostler, G., Feeney, J., Roberts, G. C. K., & Benkovic, S. J. (1989) *Biochemistry 28,* 5743−5750.

Andrews, J., Minter, S. J., & Davies, R. W. (1991) *Gene 100,* 219−224.

Antonjuk, D. J., Birdsall, B., Burgen, A. S. V., Cheung, H. T. A., Clore, G. M., Feeney, J., Gronenborn, A., Roberts, G. C. K., & Tran, T. Q. (1984) *Br. J. Pharmacol. 81,* 309.

Badii, R., Basran, J., Casarotto, M. G., & Roberts, G. C. K. (1995) *Protein Expression Purif.* (in press).

Bajorath, J., Kitson, D. H., Fitzgerald, G., Anderson, J., Kraut, J., & Hagler, A. T. (1991) *Proteins 9,* 217.

Benkovic, S. J., Fierke, C. A., & Naylor, A. M. (1988) *Science 239,* 1105−1110.

Birdsall, B., Burgen, A. S. V., & Roberts, G. C. K. (1980) *Biochemistry 19,* 3723−3731.

Birdsall, B., Gronenborn, A., Hyde, E. I., Clore, G. M., Roberts, G. C. K., Feeney, J., & Burgen, A. S. V. (1982) *Biochemistry 21,* 5831.

Birdsall, B., King, R. W., Wheeler, M. R., Lewis, C. A., Jr., Goode, R. B., Dunlap, R.B., & Roberts, G. C. K. (1983) *Anal. Biochem. 132,* 353−361.

Birdsall, B., DeGraw, J., Feeney, J., Hammond, S. J., Searle, M. S., Roberts, G. C. K., Colwell, W. T., & Crase, J. (1987) *FEBS Lett., 217,* 106.

Birdsall, B., Andrews, J., Ostler, G., Tendler, S. J. B., Feeney, J., Roberts, G. C. K., Davies, R. W., & Cheung, H. T. A. (1989a) *Biochemistry 28,* 1353−1362.

Birdsall, B., Feeney, J., Tendler, S. J. B., Hammond, S. J., & Roberts, G. C. K. (1989b) *Biochemistry 28,* 2297−2305.

Birdsall, B., Arnold, J. R. P., Jimenez Barbero, J., Frenkiel, T. A., Bauer, C. J., Tendler, S. J. B., Carr, M. D., Thomas, J. A., Roberts, G. C. K., & Feeney, J. (1990) *Eur. J. Biochem. 191,* 659−668.

Blakley, R. L., Appleman, J. R., Freisheim, J. H., & Jablonsky, M. J. (1993) *Arch. Biochem. Biophys. 306,* 501−509.

Bodenhausen, G., Kogler, H., & Ernst, R. R. (1984) *J. Magn. Reson. 46,* 269−275.

Bolin, J. T., Filman, D. J., Matthews, D. A., Hamlin, R. C., & Kraut, J. (1982) *J. Biol. Chem. 257,* 13650−13662.

Brown, K. A., & Kraut, J. (1992) *Faraday Discuss. 93,* 217−224.

Brown, S. C., Weber, P. L., & Mueller, L. (1988) *J. Magn. Reson. 77*, 166−169.
Bystroff, C., Oatley, S. J., & Kraut, J. (1990) *Biochemistry 29*, 3263−3277.
Carr, M. D., Birdsall, B., Frenkiel, T. A., Bauer, C. J., Jimenez-Barbero, J., McCormick, J. E., Feeney, J., & Roberts, G. C. K. (1991) *Biochemistry 30*, 6330−6341.
Charlton, P. A., Young, D. W., Birdsall, B., Feeney, J., & Roberts, G. C. K. (1979) *J. Chem. Soc., Chem. Commun.* 922.
Charlton, P. A., Young, D. W., Birdsall, B., Feeney, J., & Roberts, G. C. K. (1985) *J. Chem. Soc., Perkin Trans. I* 1349.
Chen, J.-T., Mayer, R. J., Fierke, C. A., & Benkovic, S. J. (1985) *J. Cell. Biochem. 29*, 73−82.
Chen, J.-T., Taira, K., Tu, C.-P. D., & Benkovic, S. J. (1987) *Biochemistry 26*, 4093−4100.
Chen, Y.-Q., Kraut, J., Blakley, R. L., & Callender, R. (1994) *Biochemistry 33*, 7021−7026.
Cheung, H. T. A., Birdsall, B., Frenkiel, T. A., Chau, D. D., & Feeney, J. (1993) *Biochemistry 32*, 6846−6854.
Cocco, L., Groff, J. P., Temple, C., Montgomery, J. A., London, R. E., & Blakley, R. L. (1981) *Biochem. Biophys. Res. Commun. 100*, 413−419.
Dann, J. G., Ostler, G., Bjur, R. A., King, R. W., Scudder, P., Turner, P. C., Roberts, G. C. K., Burgen, A. S. V., & Harding, N. G. L. (1976) *Biochem. J. 157*, 559−571.
David, C. L., Howell, E. E., Farnum, M. F., Villafranca, J. E., Oatley, S. O., & Kraut, J. (1992) *Biochemistry 31*, 9813−9822.
Davies, J. F., II, Delcamp, T. J., Prendergast, N. J., Ashford, V. A., Freisheim, J.H., & Kraut, J. (1990) *Biochemistry 29*, 9467−9479.
Dawson, R. M. C., Elliott, D. C., Elliott, W. H., & Jones, K. M. (1986) *Data for Biochemical Research* 3rd ed., Oxford University Press, Oxford, U.K.
Dion, A., Linn, C. E., Bradrick, T. D., Georghiou, S., & Howell, E. E. (1993) *Biochemistry 32*, 3479−3487.
Dunn, S. M. J., Batchelor, J. G., & King, R. W. (1978) *Biochemistry 17*, 2356−2364.
Ellis, K. J., & Morrison, J. F. (1982) *Methods Enzymol. 87*, 405−426.
Farnum, M. F., Magde, D., Howell, E. E., Hirai, J. T., Warren, M. S., Grimsley, J. K., & Kraut, J. (1991) *Biochemistry 30*, 11567−11579.
Feeney, J. (1990) *Biochem. Pharmacol. 40*, 141.
Fierke, C. A., Johnson, K. A., & Benkovic, S. J. (1987) *Biochemistry 26*, 4085−4092.
Filman, D. J., Bolin, J. T., Matthews, D. A., & Kraut, J. (1982) *J. Biol. Chem. 257*, 13663−13672.
Freisheim, J. H., & Matthews, D. A. (1984) in *Folate Antagonists as Therapeutic Agents* (Sirotnak, F. M., Burchill, J. J., Ensminger, W. B., & Montgomery, J. A., eds.) Vol. 1, pp 69−131, Academic Press Inc., New York.
Gready, J. E. (1985) *Biochemistry 24*, 4761−4766.
Hitchings, G. H., & Roth, B. (1980) in *Enzyme Inhibitors as Drugs* (Sandler, M., Ed.) pp 263−270, Macmillan, London.
Hood, K., & Roberts, G. C. K. (1978) *Biochem. J. 171*, 358.
Howell, E. E., Villafranca, J. E., Warren, M. S., Oatley, S. J., & Kraut, J. (1986) *Science 231*, 1123−1128.
Howell, E. E., Warren, M. S., Booth, C. L. J., Villafranca, J. E., & Kraut, J. (1987) *Biochemistry 26*, 8591−8598.
Huennekens, F. M., & Scrimgeour, K. G. (1964) In *Pteridine Chemistry* (Pfleiderer, W., Ed.) pp. 2297−2305, Pergamon Press, Oxford.
Jimenez, M. A., Arnold, J. R. P., Andrews, J., Thomas, J. A., Roberts, G. C. K., Birdsall, B., & Feeney, J. (1989) *Protein Eng. 2*, 627−631.
Kallen, R. G., & Jencks, W. P. (1966) *J. Biol. Chem. 241*, 5845−5850.
Li, L., Falzone, C. J., Wright, P. E., & Benkovic, S. J. (1992) *Biochemistry 31*, 7826−7833.
Maharaj, G., Selinsky, B. S., Appleman, J. R., Perlman, M., London, R. E., & Blakley, R. L. (1990) *Biochemistry 29*, 4554−4560.
Matthews, C. K., & Huennekens, F. M. (1963) *J. Biol. Chem 238*, 3436−3442.
Matthews, D. A., Alden, R. A., Bolin, J. T., Freer, S. T., Hamlin, R., Xuong, N.-H., Kraut, J., Poe, M., Williams, M., & Hoogsteen, K. (1977) *Science 197*, 452−455.
Matthews, D. A., Bolin, J. T., Burridge, J. M., Filman, D. J., Volz, K. W., Kaufman, B. T., Beddall, C. R., Champness, J. N., Stammers, D. K., & Kraut, J. (1985a) *J. Biol. Chem. 260*, 381−391.
Matthews, D. A., Bolin, J. T., Burridge, J. M., Filman, D. J., Volz, K. W., & Kraut, J. (1985b) *J. Biol. Chem. 260*, 392−399.
McTigue, M. A., Davies, J. F., II, Kaufman, B. T., & Kraut, J. (1992) *Biochemistry 31*, 7264−7273.
McTigue, M. A., Davies, J. F., II, Kaufman, B. T., & Kraut, J. (1993) *Biochemistry 32*, 6855−6862.
Oefner, C., D'Arcy, A., & Winkler, K. K. (1988) *Eur. J. Biochem. 174*, 377−385.
Orr, G. A., & Blanchard, J. S. (1984) *Anal. Biochem. 142*, 232−234.
Plateau, P., & Gueron, M. (1982) *J. Am. Chem. Soc. 104*, 7310−7311.
Poe, M. (1977) *J. Biol. Chem. 277*, 3724−3728.
Prendergast, N. J., Delcamp, T. J., Smith, P. L., & Freisheim, J. H. (1988) *Biochemistry 27*, 3664−3671.
Prendergast, N. J., Appleman, J. R., Delcamp, T. J., Blakely, R. L., & Freisheim, J. H. (1989) *Biochemistry 28*, 4645−4650.
Rabinowitz, J. C. (1960) *Enzymes, 2nd Ed. 2*, 185−252.
Rance, M., Sørensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., & Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun. 117*, 479−483.
Roberts, G. C. K. (1990a) in *Protein Engineering* (Ikehara, M., Ed.) pp 45−52, Japan Scientific Societies Press, Tokyo, Japan.
Roberts, G. C. K. (1990b) in *Chemistry and Biology of Pteridines 1989; Pteridines and Folic Acid Derivatives* (Curtius, H. C., Ghisla, S., & Blau, N., Eds.) pp 681−693, de Gruyter, Berlin.
Roberts, G. C. K., Feeney, J., Burgen, A. S. V., & Daluge, S. (1981) *FEBS Lett. 131*, 85.
Roth, B., & Strelitz, J. Z. (1969) *J. Org. Chem. 34*, 821−836.
Seeger, D. R., Cosulich, D. B., Smith, J. M., & Hultquist, M. E. (1949) *J. Am. Chem. Soc. 71*, 1753−1758.
Selinsky, B. S., Perlman, M. E., London, R. E., Unkefer, C. J., Mitchell, J., & Blakley, R. L. (1990) *Biochemistry 29*, 1290−1296.
Soteriou, A., Carr, M. D., Frenkiel, T. A., McCormick, J. E., Bauer, C. J., Sali, D., Birdsall, B., & Feeney, J. (1993) *J. Biomol. NMR 3*, 535−546.
Stammers, D. K., Champness, J. N., Beddell, C. R., Dann, J. G., Eliopoulos, E., Geddes, A. J., Ogg, D., & North, A. C. T. (1987) *FEBS Lett. 218*, 178−184.
Stone, S. R., & Morrison, J. F. (1982) *Biochemistry 21*, 3753−3765.
Stone, S. R., & Morrison, J. F. (1984) *Biochemistry 23*, 2753−2758.
Sze, K.-H., Barsukov, I. L., & Roberts, G. C. K. (1995) *J. Magn. Reson., Ser. A* (in press).
Taira, K., Chen, J.-T., Fierke, C. A., & Benkovic, S. J. (1987) *Bull. Chem. Soc. Jpn. 60*, 3025−3030.
Thillet, J., Absil, J., Stone, S. R., & Pictet, R. (1988) *J. Biol. Chem. 263*, 12500−12508.
Thillet, J., Adams, J. A., & Benkovic, S. J. (1990) *Biochemistry 29*, 5195−5202.
Thomas, J. A., Arnold, J. R. P., Basran, J., Andrews, J., Roberts, G. C. K., Birdsall, B., & Feeney, J. (1994) *Protein Eng. 7*, 783−792.
Uchimaru, T., Tsuzuki, S., Tanabe, K., Benkovic, S. J., Furukawa, K., & Taira, K. (1989) *Biochem. Biophys. Res. Commun. 161*, 64−68.
Villafranca, J. E., Howell, E. E., Voet, D. H., Strobel, M. S., Ogden, R. C., Abelson, J. N., & Kraut, J. (1983) *Science 222*, 782−788.
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.
Wagner, C. R., Thillet, J., & Benkovic, S. J. (1992) *Biochemistry 31*, 7834−7840.
Warren, M. S., Brown, K. A., Farnum, M. F., Howell, E. E., & Kraut, J. (1991) *Biochemistry 30*, 11092−11103.
Williams, E. A., & Morrison, J. F. (1992) *Biochemistry 31*, 6801−6811.

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