The influence of aspartate 26 on the tautomeric forms of folate bound to *Lactobacillus casei* dihydrofolate reductase

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Abstract **The ternary complex of *Lactobacillus casei* dihydrofolate reductase (DHFR) with folate and NADP$^+$ exists as a mixture of three interconverting forms (I, IIa and IIb) whose relative populations are pH dependent, with an effective p*K* of approx. 6. To investigate the role of Asp^{26} in this pH dependence we have measured the ^{13}C chemical shifts of [2,4*a*,7,9-$^{13}C_4$]folate in its complex with the mutant DHFR Asp^{26} → Asn and NADP$^+$. Only a single form of the complex is detected and this has the characteristics of form I, an enol form with its N1 unprotonated. A study of the pH dependence of the ^{13}C chemical shifts of DHFR selectively labelled with [4-^{13}C]aspartic acid in its complex with folate and NADP$^+$ indicates that no Asp residue has a p*K* value greater than 5.4. Two of the Asp CO_2^- signals appear as non-integral signals with chemical shifts typical of non-ionised COOH groups and with a pH dependence characteristic of the slow exchange equilibria previously characterised for signals in forms I and IIb (or IIa). It is proposed that the protonation/deprotonation controlling the equilibria involves the O4 position of the folate and that Asp^{26} influences this indirectly by binding in its CO_2^- form to the protonated N1 group of folate in forms I and IIa thus reducing the p*K* involving protonation at the O4 position to approx. 6. These findings indicate that, in forms I and IIa of the ternary complex, folate binds to DHFR in a very similar way to methotrexate.**

Key words: Dihydrofolate reductase; ^{13}C-NMR; Folate; Tautomeric form; Ionisation state

1. Introduction

Dihydrofolate reductase (DHFR) (EC 1.5.1.3) catalyses the reduction of 7,8-dihydrofolate (and folate with lower efficiency) to 5,6,7,8-tetrahydrofolate in an NADPH-linked reaction. The enzyme is of continuing pharmacological interest as the target for antifolate drugs such as trimethoprim (antibacterial) and methotrexate (anti-cancer) which act by inhibiting dihydrofolate reductase in parasitic and malignant cells [1]. There have been several detailed studies of the mechanism and kinetics of the enzyme and also extensive X-ray and NMR studies of the structures of its complexes with inhibitors and substrates (reviewed in [1–5]).

Studies of the stereochemistry of the reduction of folate and dihydrofolate showed that the orientation of the pteridine ring of the substrate in the catalytically functional complex differs by approx. 180° from that of the inhibitor methotrexate [6–8] and this was confirmed by X-ray diffraction studies of complexes of folate with mammalian and bacterial dihydrofolate reductases [9–11]. NMR studies have also revealed that the ternary complex of *Lactobacillus casei* DHFR with folate and NADP$^+$ exists as a mixture of three interconverting forms whose relative populations are pH-dependent [12–14]. Two of the forms (designated forms I and IIa) have the same pteridine ring orientation as that observed in the DHFR-methotrexate complex while the third (form IIb) has the pteridine ring turned over by approx. 180°. The latter orientation is referred to as the 'active' conformation since this orientation would lead to the observed stereochemistry for the enzyme-catalysed reduction. Recent NMR studies of specifically ^{13}C-labelled folate [15] have indicated that in forms I and IIa of the DHFR-folate-NADP$^+$ complex the pterin is in the enolic tautomeric form, whereas in the 'active' form IIb it is in the keto form, as observed for uncomplexed folate at pH 5.5 (see Fig. 1). Proposals for the catalytic mechanism of DHFR have suggested that keto-enol tautomerism of the substrate may be involved [11,16] giving added interest to the study of these equilibria in the DHFR-folate-NADP$^+$ complex.

The variation in the relative proportions of forms I, IIa and IIb of this complex as a function of pH has been studied using ^{1}H-, ^{3}H-, ^{13}C-, ^{15}N- and ^{31}P-NMR measurements on the complex formed with wild-type enzyme [12–14]. ^{1}H studies of the complex formed with the Asp^{26} → Asn mutant enzyme showed that it exists in only one conformational form (resembling form I) and no spectral changes were observed on changing the pH over the range 5.4–7.0 [17]. This clearly implicated Asp^{26} (a residue conserved in all bacterial dihydrofolate reductases) in the pH dependence of the conformational equilibria observed in complexes with the wild-type enzyme, and it was suggested that this pH dependence might arise from the protonation and deprotonation of Asp^{26}.

We now report further ^{13}C-NMR studies of the *L. casei* DHFR-folate-NADP$^+$ complex, using both ^{13}C-labelled substrate and enzyme, aimed at establishing clearly the origin of the pH dependence of the conformational equilibrium and the tautomeric and ionisation states of the substrate in this complex.

2. Materials and methods

The preparation and purification of [2,4*a*,7,9-$^{13}C_4$]folate [15], of the Asp^{26} → Asn mutant of *L. casei* DHFR [17] and of [4-^{13}C]Asp-labelled wild-type DHFR [18] have been described previously. Folate and NADP$^+$ were obtained from Sigma Chemical Co. The complex of [2,4*a*,7,9-$^{13}C_4$]folate and NADP$^+$ with DHFR was prepared as described previously [15]. The complex of folate and NADP$^+$ with

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[4-^{13}C]Asp-DHFR was formed by adding 2 molar equivalents of each ligand to ensure that the enzyme was wholly in the form of the DHFR-folate-NADP$^+$ complex; the enzyme concentration (determined by absorbance measurements) was 0.5–0.7 mM. The NMR samples were dissolved in 90% H_2O/10% ^2H_2O containing 50 mM potassium phosphate and 200 mM KCl. pH adjustments were made by making 2 μl additions of concentrated HCl to the protein solutions and measuring the pH using a Corning Model 155 pH meter. The pH values are meter readings uncorrected for the small isotope effect due to the 10% ^2H_2O. NMR spectra were recorded at 293K using Bruker AMX600 and Varian 400 NMR spectrometers. The ^{13}C spectra were referenced to external tetramethylsilane using methods described by Cheung and co-workers [15].

3. Results and discussion

In our earlier studies of the DHFR-folate-NADP$^+$ complex, we showed that the pH dependence of the relative populations of the three forms of the complex could be accounted for either by the simple ionisation of a group with p$K \sim 6$ or by a model in which an ionisable group has p$K < 5$ in forms IIa and IIb, and p$K > 7$ in form I [13,14]. In order to test the hypothesis that this ionisable group might be that of Asp^{26} [14,17], we have used ^{13}C-NMR to examine directly the charge states of the aspartic acid residues of the enzyme in the complex with the wild-type enzyme and the tautomeric and ionisation states of folate in the ternary complex with the Asp$^{26} \rightarrow$ Asn mutant.

3.1. Ionisation states of the aspartic acid residues

The ^{13}C-NMR spectra of the ternary complex of folate and NADP$^+$ with DHFR selectively labelled with [4-^{13}C]aspartic acid were measured as a function of pH over the range pH 4.6–7.0. Fig. 2 shows representative spectra obtained for the complex at different pH values and Fig. 3 displays the pH dependence of the chemical shifts of the Asp γ-carbon (carboxyl) resonances. As one would expect for Asp residues, the ^{13}C chemical shifts of most of the signals are dependent on pH over the range pH 4.6–6.2, but none of them show chemical shift changes over the range pH 6.2–7.0. Some signals around 176 ppm arise from ^{13}C at natural abundance present in non-enriched residues in the protein. In studies on *Escherichia coli* ribonuclease H, the ^{13}C signals of Asp γ-carbons were found to appear between 176 and 179 ppm in the deprotonated state and between 173 and 175 ppm in the protonated state [19].

Although the specific assignments of these resonances to the 15 Asp residues of the protein, and particularly the assignment of the Asp^{26} resonance[2] are not known, several useful observations can be made. We can predict the behaviour expected of the resonance of Asp^{26} if this is indeed the residue whose ionisation controls the pH dependence of the conformational equilibrium between forms I and (IIa+IIb). In the simplest model, it would have a pK of ~ 6 [13,14], but this can be eliminated since the pH dependence of the γ-^{13}C resonances indicates that no Asp residue has a pK greater than 5.4. In the alternative model the chemical shift in form I, which predominates at low pH and has p$K > 7$, should be characteristic of the carboxyl (-CO_2H) state, while in forms IIa and IIb, which predominate at high pH and have p$K < 5$, the chemical shift will be that of the carboxylate ($-CO_2^-$) state; interconversion between these forms is slow, so that changes in resonance intensity, rather than chemical shift, as a function of pH are expected.

It can be seen in Figs. 2 and 3 that three γ-carbon ^{13}C signals show no change in chemical shift over the range pH 4.6–7.0: one of these (at 175.7 ppm) is present over the complete pH range whereas two others (179.0 and 177.9 ppm) are of non-integral intensity and are detectable only over parts of the range. The ^{13}C signal at 175.7 ppm is present at the same position and with the same intensity over the complete titration range; it is therefore unlikely to arise from a residue involved in the conformational equilibria in the DHFR-folate-NADP$^+$ complex. It may arise from the carboxylate carbon of Asp^{25} which forms a salt bridge with His^{153} [20]; the pK of His^{153} is >8.0 [21], and one would correspondingly expect a low pK for Asp^{25}. Of the remaining resonances, that at 177.9 ppm is seen only between pH 5.6 and 7.0 its intensity decreasing as the pH decreases, whereas in contrast the resonance at 179.0 ppm is seen only at low pH values and its intensity decreases as the pH increases from pH 4.6 to 5.1. Although this is the behaviour expected for the changes in intensity of the Asp^{26} resonance in form IIa or IIb (177.9 ppm signal), and in form I (179.0 ppm signal) the chemical shifts of both of these signals are in the range expected for an unprotonated $^{13}CO_2^-$ [19].

These ^{13}C experiments thus indicate that in the low pH form (form I) of the complex Asp^{26} remains unprotonated over the pH range in which this form is observable[3]. The same is, however, true for Asp^{26} in the high pH state of the complex (forms IIa and IIb). Thus, although the effects of the Asp$^{26} \rightarrow$ Asn mutation [17] clearly implicate Asp^{26} in the pH dependence of the conformational equilibria in the DHFR-folate-NADP$^+$ complex, it cannot be involved directly, since it has the same carboxylate charge state in the different forms of the complex.

3.2. The ionisation and tautomeric states of folate in its complex with NADP$^+$ and the Asp$^{26} \rightarrow$ Asn DHFR mutant

In order to understand the effects of the Asp$^{26} \rightarrow$ Asn mutation on the conformational equilibria, the tautomeric and ionisation states of the bound folate in the ternary Asp$^{26} \rightarrow$ Asn DHFR-folate-NADP$^+$ complex have been examined by studying the ^{13}C spectra of the complex formed with [2,4*a*,7,9-$^{13}C_4$]folate.

The ^{13}C spectra of this ternary complex were recorded at pH 5.7 and pH 7.0 (see Fig. 4a,b). At each pH, a single signal was detected for each labelled site, corresponding to the presence of a single conformational form, and there were no differences in chemical shift or relative intensity of the signals at the two pH values. A comparison of these chemical shifts with those observed previously for [2,4*a*,7,9-$^{13}C_4$]folate in its ternary complex with NADP$^+$ and wild-type DHFR (shown in Fig. 4c) confirmed that the single form of the complex with

[2] We have attempted to assign the Asp^{26} signal by examining a complex of folate with the mutant Asp$^{26} \rightarrow$ Asn DHFR labelled with [4-^{13}C]aspartic acid: the experiments indicated that the Asp^{26} signal does not fall outside of the overlapped region 177.0 to 179.5 ppm.

[3] This is supported by the ^{1}H chemical shifts of the α- and β-protons of Asp^{26} in form I [15] which are almost identical to those in the DHFR-methotrexate complex where Asp^{26} is thought to remain unprotonated at pH 5 [22].

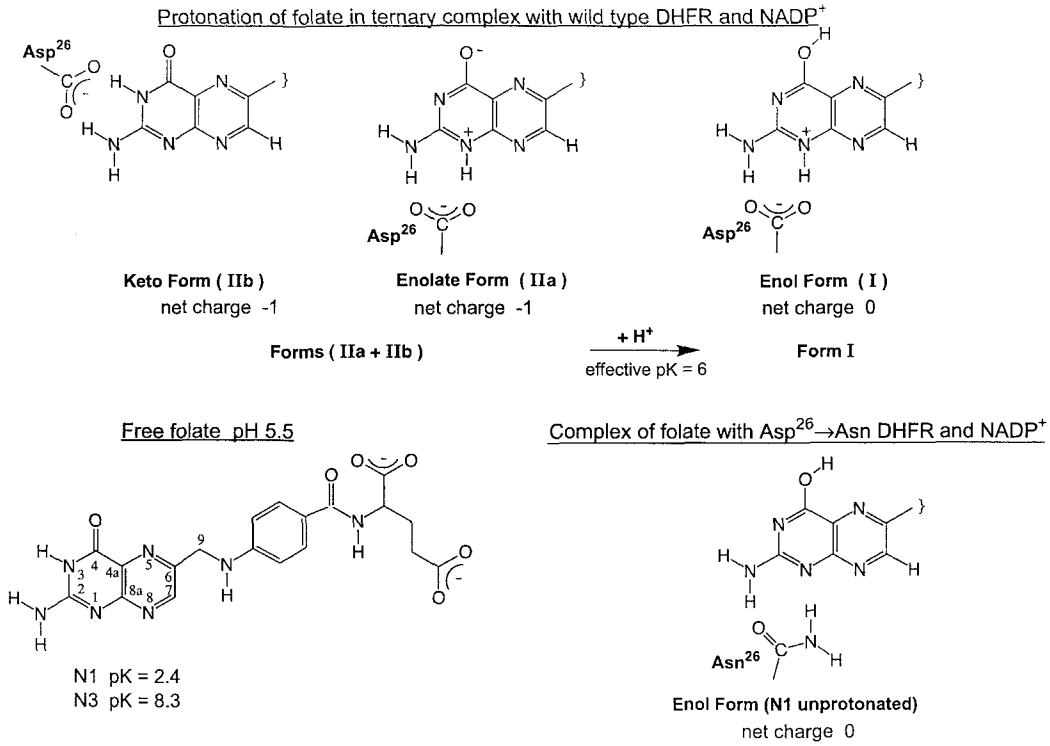

Fig. 1. The structures and tautomeric forms of folate: free at pH 5.5, complexed to wild-type DHFR and $NADP^+$ (forms I, IIa and IIb) and complexed to $Asp^{26} \rightarrow Asn$ DHFR and $NADP^+$. The net charges of the complexes are indicated.

$Asp^{26} \rightarrow Asn$ DHFR most closely resembles form I of the wild-type complex, in agreement with the conclusions from the earlier ^1H studies [17].

Cheung and co-workers [15] have estimated the ^{13}C chemical shifts for various tautomeric states of folate and Fig. 4 includes a schematic diagram showing the predicted ^{13}C chemical shifts for the C2 and C4a carbons in various tautomeric forms; estimated shifts were not obtained for the C7 carbons due to the lack of suitable model compounds [15].

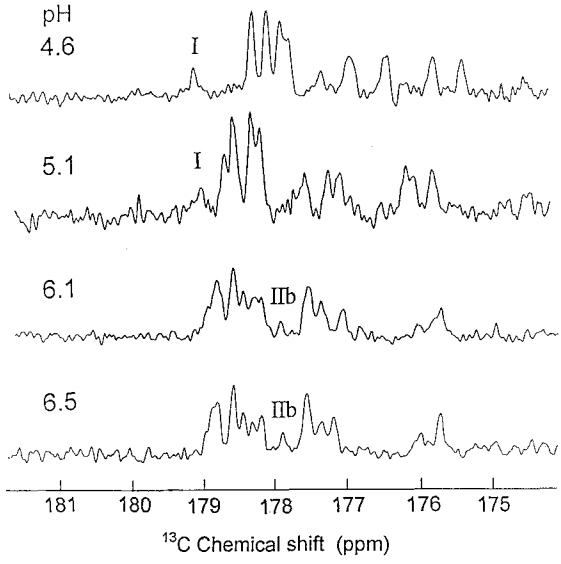

Fig. 2. Part of the 150.8 MHz ^{13}C-NMR spectrum at 293 K of the 1:1:1 complex of folate and $NADP^+$ with [4-^{13}C]Asp DHFR at pH values (a) 4.6, (b) 5.1, (c) 6.1 and (d) 6.5. The resonances assigned to Asp^{26} in forms I and IIb of the complex are indicated.

The ^{13}C chemical shift most characteristic of form I is that of C4a (124.5 ppm in the $Asp^{26} \rightarrow Asn$ DHFR complex and 123.9 ppm in the wild-type DHFR complex). These values compare favourably with the predicted ^{13}C chemical shifts for C4a in various models of the enol tautomer of folate (123.3 to 125.7 ppm; Fig. 4), whereas the C4a chemical shifts in forms IIa and IIb of the wild-type complex are 130 ± 2 and 129.5 ppm, respectively. The chemical shift of the C2 carbon (162.3 ppm) in the ternary complex with $Asp^{26} \rightarrow Asn$ DHFR is somewhat further downfield than the value observed for form I in the wild-type ternary complex (159.8 ppm). Consideration of the estimated chemical shifts for various models shown schematically in Fig. 4 indicates that this shift differ-

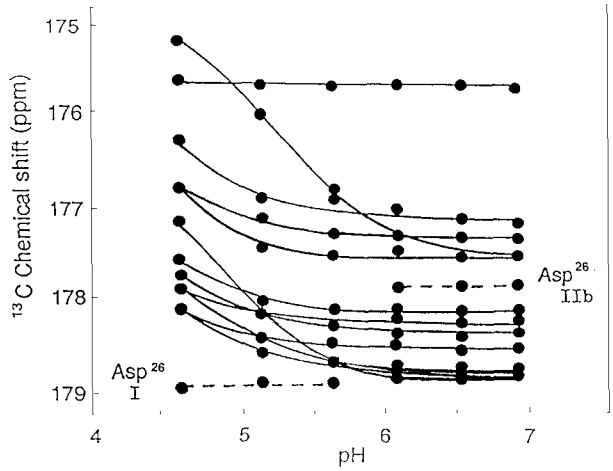

Fig. 3. The pH titration curves for the ^{13}C chemical shifts of the [4-^{13}C]Asp residues in the 1:1:1 complex of folate and $NADP^+$ with [4-^{13}C]Asp DHFR at 293 K.

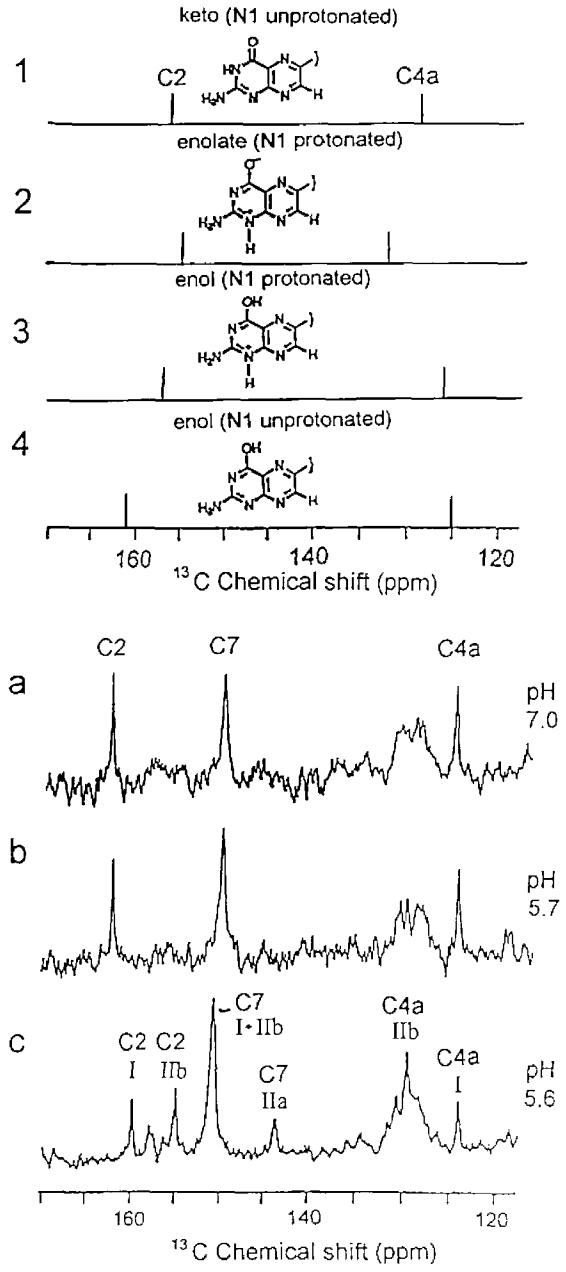

Fig. 4. Low-field region of the 100.6 MHz ^{13}C-NMR spectra of [2,4*a*,7,9-$^{13}C_4$] folic acid in its 1:1:1 ternary complex with NADP$^+$ and *L. casei* DHFR (a) formed with Asp^{26}→Asn DHFR at pH 7.0 and 281 K; (b) formed with Asp^{26}→Asn DHFR at pH 5.7 and 281 K, and (c) formed with the wild-type DHFR at pH 5.6 and 281 K (the latter taken from Cheung and co-workers [15]). The figure also includes a schematic diagram showing the structures of various keto and enolic forms of folate with their predicted ^{13}C chemical shifts for the C2 and C4a carbons (from Cheung and co-workers [15]).

ence could be explained if N1 of folate is unprotonated in form I of the Asp^{26}→Asn DHFR complex but protonated in form I of the wild-type DHFR complex (see Fig. 1), the estimated shift for an N1 unprotonated enol model being 161.4 ppm.

Cheung and co-workers [15] concluded that for the ternary wild-type DHFR complex in form IIb folate is present as the keto tautomer with N1 unprotonated, while in form IIa, folate is most probably present as the enolate tautomer with N1 protonated (Fig. 1). These two forms thus differ only in the position of a proton on the pterin ring – on N1 in form IIa, and on N3 in form IIb – explaining the observation that the IIa/IIb ratio is independent of pH. This tautomerisation is accompanied by a change in the orientation of the pterin ring, so that the Asp^{26} carboxylate can form hydrogen bonds to N1-H and 2-NH_2 in form IIa, and to N3-H and 2-NH_2 in form IIb. These assignments of the tautomeric and ionisation states of folate imply that there is a difference of one proton between the folate structure in form I and those in forms IIa and IIb in the ternary complex with the wild-type DHFR (Fig. 1). This would provide a simple explanation for the observed pH dependence of the relative populations of forms I and II.

If this is the origin of the pH dependence, then there must be a mechanism by which the presence of Asp^{26} as a carboxylate anion throughout the pH range (pH 5 to 7) influences the ionisation of the folate molecule so that the effective pK of the folate is approx. 6 in the wild-type enzyme but >8 in the Asp^{26}→Asn mutant. The most likely mechanism is through an effect on the pK of N1 of folate. In free folate the pK associated with protonation on N1 is 2.4, but, in its complex with DHFR and NADP$^+$, the proximity of the carboxylate of Asp^{26} and the possibility of ion-pair formation increases this pK so that N1 is protonated in forms I and IIa. This protonation on N1 has the effect of decreasing the pK for loss of a proton from N3/O4 from the value of 8.3 in free folate (for deprotonation of N3 in the keto tautomer [23]) to approx. 6 in the DHFR-folate-NADP$^+$ complex. In the case of Asp^{26}→Asn DHFR, the replacement of the carboxylate by a carboxamide favours binding of folate unprotonated at N1 (Fig. 1), and the pK of O4 in this species is sufficiently high that deprotonation of the enolic group is not observed in the accessible pH range.

These results thus indicate that the binding of folate in form I of the DHFR-folate-NADP$^+$ complex (see Fig. 1) closely parallels the binding of the inhibitor methotrexate: the orientation in the binding site is the same, and methotrexate is also protonated on N1 when bound to the wild-type enzyme but unprotonated when bound to the Asp^{26}→Asn mutant (see Fig. 1) [16,24]. There is a further parallel between the DHFR-folate-NADP$^+$ complex and the catalytically functional DHFR-dihydrofolate-NADPH complex, since in both cases Asp^{26} appears to determine the pH dependence indirectly, through its influence on the pK of groups in the pterin ring (N1 and O4 in the case of folate, N5 in the case of dihydrofolate) [11,16,25].

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References

[1] Blakley, R.L. (1985) in: Folates and Pterins, vol. 1 (Blakley, R.L. and Benkovic, S.J. eds.) pp. 191–253, Wiley, New York.
[2] Roberts, G.C.K. (1983) in: Chemistry and Biology of Pteridines (Blair, J.A. ed.) pp. 197–214, De Gruyter, Berlin.
[3] Freisheim, J.H. and Matthews, D.A. (1984) in: Folate Antagonists as Chemotherapeutic Agents, vol. 1 (Sirotnak, F.M., Burchall, J.J., Ensminger, W.B. and Montgomery, J.A. eds.) pp. 69–131, Academic Press, New York.

[4] Beddell, C.R. (1984) in: X-Ray Crystallography and Drug Action (Horn, A.S. and De Ranter, C.J. eds.) pp. 169–193, Clarendon Press, Oxford.
[5] Feeney, J. (1990) Biochem. Pharmacol. 40, 141–152.
[6] Hitchings, G.H. and Roth, B. (1980) in: Enzyme Inhibitors as Drugs (Sandler, M. ed.) pp. 263–270, Macmillan, London.
[7] Charlton, P.A., Young, D.W., Birdsall, B., Feeney, J. and Roberts, G.C.K. (1979) J. Chem. Soc. Chem. Commun. 922–924.
[8] Charlton, P.A., Young, D.W., Birdsall, B., Feeney, J. and Roberts, G.C.K. (1985) J. Chem. Soc. Perkin Trans. 1, 1349–1353.
[9] Oefner, C., D'Arcy, A. and Winkler, F.K. (1988) Eur. J. Biochem. 174, 377–385.
[10] Bystroff, C., Oatley, S.J. and Kraut, J. (1990) Biochemistry 29, 3263–3277.
[11] Brown, K.A. and Kraut, J. (1992) Faraday Disc. 93, 217–224.
[12] Birdsall, B., Gronenborn, A.M., Clore, G.M., Roberts, G.C.K., Feeney, J. and Burgen, A.S.V. (1981) Biochem. Biophys. Res. Commun. 101, 1139–1144.
[13] Birdsall, B., Gronenborn, A.M., Hyde, E.I., Clore, G.M., Roberts, G.C.K., Feeney, J. and Burgen, A.S.V. (1982) Biochemistry 21, 5831–5838.
[14] Birdsall, B., Feeney, J., Tendler, S.J.B., Hammond, S.J. and Roberts, G.C.K. (1989) Biochemistry 28, 2297–2305.
[15] Cheung, H.T.A., Birdsall, B., Frenkiel, T.A., Chau, D.D. and Feeney, J. (1993) Biochemistry 32, 6846–6854.
[16] Basran, J., Casarotto, M.G., Barsukov, I., Badii, R. and Roberts, G.C.K. (1995) Biochemistry 34, 2872–2882.
[17] Jimenez, M.A., Arnold, J.R.P., Andrews, J., Thomas, J.A., Roberts, G.C.K., Birdsall, B. and Feeney, J. (1989) Protein Eng. 2, 627–631.
[18] Badii, R., Basran, J., Casarotto, M.G., Roberts, G.C.K. (1995) Protein Express. Purif. 6, 237–243.
[19] Oda, Y., Yamazaki, T., Nagayama, K., Kanaya, S., Kuroda, Y. and Nakamura, H. (1994) Biochemistry 33, 5275–5284.
[20] Bolin, J.T., Filman, D.J., Matthews, D.A., Hamlin, R.C. and Kraut, J. (1982) J. Biol. Chem. 257, 13650–13662.
[21] Wyeth, P., Gronenborn, A., Birdsall, B., Roberts, G.C.K., Feeney, J. and Burgen, A.S.V. (1980) Biochemistry 19, 2608–2615.
[22] Cocco, L., Groff, J.P., Temple Jr., C., Montgomery, J.A., London, R.E., Matwiyoff, N.A. and Blakley, R.L. (1981) Biochemistry 20, 3972–3978.
[23] Von Dieffenbacher, A., Mondelli, R. and Von Philipsborn, W. (1966) Helv. Chim. Acta 49, 1355–1377.
[24] Howell, E.E., Villafranca, J.E., Warren, M.S., Oatley, S.J. and Kraut, J. (1986) Science 231, 1123–1128.
[25] Chen, Y.Q., Kraut, J., Blakley, R.L. and Callender, R. (1994) Biochemistry 33, 7021–7026.