13C NMR studies of complexes of Escherichia coli dihydrofolate reductase formed with methotrexate and with folic acid

ARTICLE *in* FEBS LETTERS · DECEMBER 1992

Impact Factor: 3.17 · DOI: 10.1016/0014-5793(92)80923-5 · Source: PubMed

3 AUTHORS, INCLUDING:

H.T. A. Cheung
University of Sydney
95 PUBLICATIONS **1,268** CITATIONS
SEE PROFILE

James Feeney
MRC National Institute for Medical Research
315 PUBLICATIONS **7,818** CITATIONS
SEE PROFILE

Volume 312, number 2,3, 147–151 FEBS 11714 November 1992

^{13}C NMR studies of complexes of *Escherichia coli* dihydrofolate reductase formed with methotrexate and with folic acid

H.T.A. Cheung[a,b], B. Birdsall[a] and J. Feeney[a]

[a]*Laboratory of Molecular Structure, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK* and [b]*Department of Pharmacy, University of Sydney, Sydney, NSW 2006, Australia*

Received 15 September 1992

^{13}C NMR studies of ^{13}C-labelled ligands bound to dihydrofolate reductase provide (DHFR) a powerful means of detecting and characterizing multiple bound conformations. Such studies of complexes of *Escherichia coli* DHFR with [4,7,8a,9-^{13}C]- and [2,4a,6-^{13}C]methotrexate (MTX) and [4,6,8a-^{13}C]- and [2,4a,7,9-^{13}C]folic acid confirm that in the binary complexes, MTX binds in two conformational forms and folate binds as a single conformation. Earlier studies on the corresponding complexes with *Lactobacillus casei* DHFR indicated that, in this case, MTX binds as a single conformation whereas folate binds in multiple conformational forms (both in its binary complex and ternary complex with NADP$^+$); two of the bound conformational states for the folate complexes are very different from each other in that there is a 180° difference in their pteridine ring orientation. In contrast, the two different conformational states observed for MTX bound to *E. coli* DHFR do not show such a major difference in ring orientation and bind with N1 protonated in both forms. The major difference appears to involve the manner in which the 4-NH_2 group of MTX binds to the enzyme (although the same protein residues are probably involved in both interactions). Addition of either NADP$^+$ or NADPH to the *E. coli* DHFR–MTX complex results in a single set of ^{13}C signals for bound methotrexate consistent with only one conformational form in the ternary complexes.

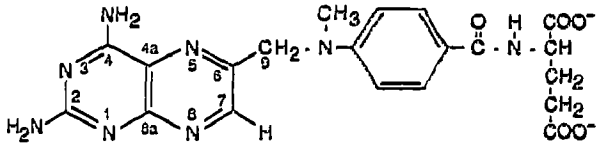
Dihydrofolate reductase; Methotrexate; Folic acid; ^{13}C NMR; Multiple conformations

1. INTRODUCTION

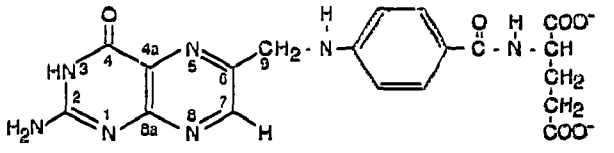
Dihydrofolate reductase (DHFR) (EC 1.5.1.3) catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate [1]. The enzyme is of considerable pharmacological importance, being the target for antifolate drugs such as methotrexate (MTX, an anti-neoplastic agent) and trimethoprim (an antibacterial drug). NMR studies of dihydrofolate reductase from various sources have uncovered several examples of multiconformational behaviour [2–10]. For example, Falzone et al. [6] have used ^{1}H NMR measurements to show that *Escherichia coli* DHFR exists as two conformations in solution, both in its free form and also when complexed to methotrexate. Huang and co-workers [7] have characterized further the different conformations by the use of ^{15}N NMR spectra. By contrast, *E. coli* DHFR occurs only as a single conformation in its complex with folate [7,11]. This behaviour differs from that observed for the corresponding complex formed with *Lactobacillus casei* DHFR, which exists as a single conformation in its MTX complex [12], but occurs in at least two conformations in its binary complex with folate and three conformations in the ternary complex with folate and NADP$^+$ [4].

Correspondence address: J. Feeney, Laboratory of Molecular Structure, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK.

It is important to understand the structural differences in the various complexes which underlie the interspecies differences in their ability to form multiple conformations. Such information could eventually assist in the design of more selective inhibitors. A convenient method for studying such systems is to examine the ^{13}C NMR spectra of enzyme complexes formed with selectively ^{13}C-enriched ligands. Here we report on the ^{13}C NMR spectra of complexes of [4,7,8a,9-^{13}C]- and [2,4a,6-^{13}C]MTX (scheme) and [4,6,8a-^{13}C]- and [2,4a,7,9-^{13}C]folic acid (scheme) formed with *E. coli*



(1) Methotrexate



(2) Folic Acid

Schemes 1 and 2

DHFR, and compare the results with those obtained previously for the corresponding complexes formed with *L. casei* DHFR [12,13].

2. MATERIALS AND METHODS

The methods used to synthesize the ^{13}C-labelled MTX have been reported [4]. [4,6,8a-^{13}C]- and [2,4a,7,9-^{13}C]folic acid were made from [4,6,8a-^{13}C]- and [2,4a,7,9-^{13}C]2,4-diamino-6-methylpteridine, respectively, using procedures to be described elsewhere [13]. $NADP^+$ was obtained from Sigma Chemical Co. *E. coli* DHFR was a generous gift from Dr. J.F. Morrison: it was prepared using a strain of *E. coli* produced by mutagenesis of the DNA of the plasmid pJFM and purified using the methods described previously by Rood and Williams [15].

The samples used for the NMR experiments were prepared by dissolving lyophilized *E. coli* DHFR in ^2H_2O (^1H experiments) or 10% ^2H_2O in H_2O (^{13}C experiments) containing approximately 1 equ. of ligand, to give 1.8 ml of the final solution containing 0.5–0.7 mM DHFR, 100 or 500 mM potassium chloride and 50 or 100 mM potassium phosphate (the lower salt contents were used for experiments on the complexes with folates). Some samples were further dialyzed to remove excess ligand. Changes in pH were accomplished by making μl additions of ^2HCl or HCl (0.1 M), or NaO^2H or NaOH (0.1 M) and the pH was measured using a Radiometer Model 28 pH meter. The pH values quoted are meter readings uncorrected for any deuterium isotope effects. Dioxan (0.1%) was used as a chemical shift reference (67.5 ppm from TMS at 298 K).

Most of the ^{13}C NMR experiments were carried out at 100.4 MHz on a Bruker AM400 spectrometer at 281 and 298 K: other experiments were performed using Bruker AM500 and Varian Unity 600 spectrometers. Composite pulse decoupling of protons was used in most of the

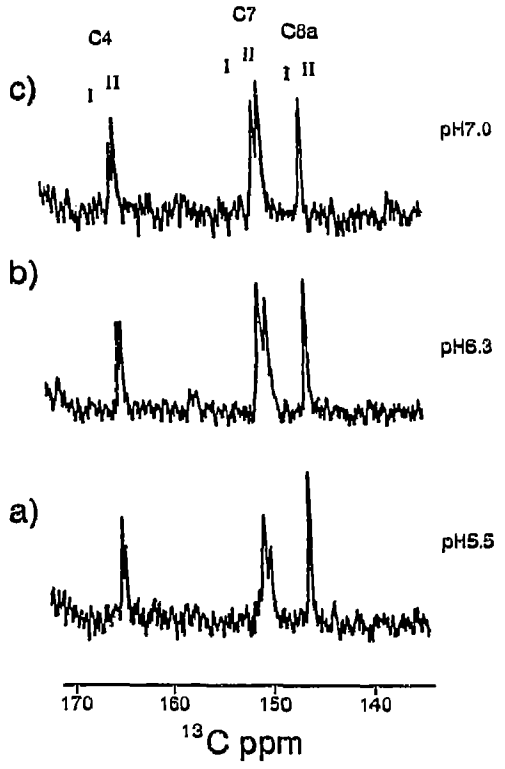


Fig. 1. ^{13}C NMR spectra of the *E. coli* DHFR binary complex formed with [4,7,8a,9-^{13}C]methotrexate measured at different pH values. Spectra were obtained at 298 K and 125 MHz (C9 region not shown).

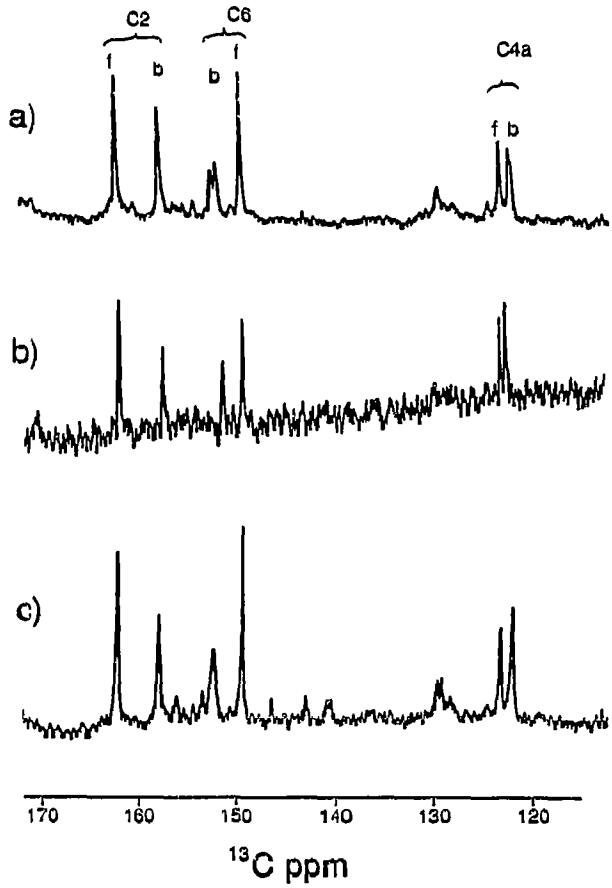


Fig. 2. ^{13}C NMR spectra of complexes of *E. coli* DHFR formed with (a) [2,4a,6-^{13}C]methotrexate (pH 6.4, 100 MHz), (b) [2,4a,6-^{13}C]methotrexate and NADPH (pH 6.5, 150 MHz), (c) [2,4a,6-^{13}C]methotrexate and $NADP^+$ (pH 6.5, 100 MHz). Spectra were obtained at 298 K. f and b refer to signals of free and bound methotrexate, respectively.

^{13}C NMR experiments [16]. The HMQC NOESY experiment was carried out on the Bruker AM500 using the sequence proposed by Gronenborn and co-workers [17].

3. RESULTS AND DISCUSSION

3.1. E. coli *DHFR–MTX complex*

The ^{13}C NMR spectra for the complexes of DHFR with [4,7,8a,9-^{13}C]- and [2,4a,6-^{13}C]MTX have been measured over the pH range 5.0–7.2. The assignments of the ^{13}C NMR spectrum of free MTX at pH 2.0 and 6.5 have already been reported [12], and transferring these assignments to those of the bound ^{13}C signals in all the complexes was relatively straightforward because of the distribution of the labels in the two analogues (see Table I). In the ^{13}C spectra of the binary complexes (shown in Figs. 1 and 2a) there is evidence for two conformations of the complex at all pH values examined, as was reported previously in the ^1H [6] and ^{15}N NMR studies [7]. For example, in the ^{13}C NMR spectra

of the binary complexes with [4,7,8a,9-^{13}C]- and [2,4a,6-^{13}C]MTX shown in Figs. 1 and 2a, 'doublet' signals are seen for C4 (0.4 ppm), C7 (0.7 ppm), C6 (0.5 ppm) and C4a (0.3 ppm), whereas C2, C8a and C9 show very little or no 'splitting'. (Huang et al. [7] reported a 2.2 ppm shift difference for N5 in the ^{15}N NMR spectra of the two forms.) The chemical shifts of the bound species do not change appreciably with pH, but the relative amounts of the two forms change; the two forms are equally present at pH 6.8 ± 0.3. This is a similar result to that reported by Huang et al. [7] in their ^{15}N studies, although they reported a somewhat lower pH value for the appearance of equal populations (pH 6).

Huang et al. [7] designated as Form I and Form II those forms which have, respectively, lower and higher populations at high pH, and we have followed their designation in assigning the two forms to their appropriate bound ^{13}C shifts (see Table I). HMQC and HMQC NOESY experiments allowed us to connect the C7 carbon shifts to the corresponding H7 proton shifts: thus the high field H7 proton signal (reported in Falzone et al. [6]) corresponds to Form I, the form favoured at low pH. Because we have analogues of MTX with all the pteridine ring carbons labelled with ^{13}C it is possible to monitor interactions between the protein and various positions on the pteridine ring. For example, the ^{13}C chemical shifts of C2 and C8a in the two bound forms indicate that N1 is protonated in both forms of the *E. coli* DHFR–MTX complex. Thus the bound ^{13}C chemical shifts of C2 and C8a (see Table I) are seen to be close to the values for free MTX at low pH, which is protonated at N1 (and very similar to the value found for bound C2 in MTX complexes with *L. casei* DHFR [13,18] where only one set of bound ^{13}C signals is observed). The C2 carbons in both forms of the *E. coli* DHFR–MTX complex have an additional chemical shift contribution of 1.4 ppm over that for protonated free MTX: a similar additional shift contribution was seen in the ^{13}C spectrum of the *L. casei* DHFR–MTX complex, and this indicates that the N1 proton is binding in a similar manner in the different complexes. It seems likely that for the *E. coli* DHFR–MTX complexes in solution, the 2-NH_2 group and the protonated N1 are directly interacting with the carboxylate group of Asp^{27}, as was found by X-ray analysis for the complex in the crystalline state [19]. This is in full agreement with the finding of Falzone et al. [6], namely, that the MTX pteridine ring has the same general orientation in the two forms (that is, one of them is not turned over with respect to the other) and that the orientation is similar to that observed in the X-ray structure.

The observed ^{13}C chemical shift differences between Forms I and II are not sufficiently large to indicate a difference in the structure of the pteridine ring in the two forms. The differences probably arise from different shielding interactions with the protein. Some contribution to the shielding differences could also arise from different interactions associated with conformational

Table I

^{13}C chemical shifts of methotrexate and folate, free and complexed with *E. coli* and *L. casei* DHFR[a]

Complex	Source	C2	C4	C4a	C6	C7	C8a	C9
DHFR–MTX	*E. coli* (I)	158.1	165.3	122.3	152.3	150.7	146.1	57.2
	E. coli (II)	158.1	164.9	122.6	152.8	150.0	146.1	57.2
DHFR–MTX	*L. casei*	157.7	164.6	124.0	151.7	148.6	145.9	56.4
Free MTX pH 2.1		156.7	164.1	123.2	152.8	150.2	145.7	56.0
DHFR–MTX–NADPH	*E. coli*	157.9	164.3	122.9	151.9	149.1	145.8	57.3
DHFR–MTX–NADPH	*L. casei*	157.7		124.2	150.8	147.7		57.3
DHFR–MTX–$NADP^+$	*E. coli*	158.2	164.8	122.3	152.7	150.4	146.4	56.4
DHFR–MTX–$NADP^+$	*L. casei*	157.9	164.6	123.3	151.7	146.6	146.9	55.1
DHFR–folate	*E. coli*	154.9	164.6	127.1	147.8	152.2	158.0	46.1
DHFR–folate	*L. casei* (IIb)	154.7	164.2	128.1	147.9	150.3	158.0	46.7
Free folate pH 5.5		155.6	165.8	128.5	151.3	150.1	154.8	47.0
DHFR–folate–$NADP^+$	*E. coli*	154.6	163.5	128.4	145.9	151.6	158.7	45.7
DHFR–folate–$NADP^+$	*L. casei* (IIb)	154.9	163.3	129.5	145.2	150.6	158.9	46.1

[a]Folate spectra were recorded at 281 K and methotrexate spectra at 298 K. The chemical shifts listed are for samples with a pH in the range 6–7; chemical shifts do not change by more than 0.2 ppm over the entire range of pHs studied (see text). Chemical shifts are referenced to dioxan = 67.5 ppm from TMS at 298 K and 67.3 ppm at 281 K.

differences involving the C6–C9 torsion angle between the two forms.

Some general conclusions about the structure of the two forms of the *E. coli* DHFR–MTX complex emerge from consideration of the available data:

(i) The 2-NH_2 group and the protonated N1 of MTX appear to bind similarly in the two forms: the similarity of the C2 and C8a bound chemical shifts indicates that the protein interactions with this part of the pteridine ring are very similar in the two forms.

(ii) The significant shift differences seen for C4, C4a, N5 and C6 are consistent with the C4 region of the pteridine ring binding differently in the two forms. These different interactions could result if the pteridine ring pivots about the N1/C2 region such that it has a different tilt angle in the two forms, placing the C4 region in different environments. This could result from differences in the interactions between the 4-NH_2 group and the interacting residues on the protein (viz. the peptide carbonyl oxygens of Ile^5 and Ile^{94}, which form hydrogen bonds with the hydrogens of the 4-NH_2 group [19]). Such different interactions would be consistent with the observations of Falzone et al. [6] who observed multiple signals for protons from Ile^5 and Ile^{94} residues in the ^1H spectrum of the *E. coli* DHFR–MTX complex, with small shift differences between the signals from the two forms. These different interactions at the 4-NH_2 group could change the overall position of the C4 region of the pteridine ring without substantially perturbing the protein interactions with the part of the ring near C2 in the two forms.

(iii) Falzone et al. [6] noted doubling of ^1H signals in the apoenzyme for several residues, including Val^{93}, Leu^{110}, Tyr^{111} and Leu^{112}. All of these residues are near to either Ile^5 or Ile^{94}, the residues we have implicated in different modes of binding to the MTX 4-NH_2 protons in the different forms. Thus the multiple conformations seen in the free enzyme appear to be maintained on forming the binary complex with MTX, and these could give rise to the different interactions with the 4-NH_2 group as reflected by the ^{13}C data.

(iv) Falzone et al. [6] noted ^1H shift differences between signals from Leu^{25} and Leu^{54} in the two forms of the DHFR–MTX complex: these results suggest that there is a difference in the orientation of the benzoyl ring in the two forms and this could contribute to the differences in ^{13}C shielding seen at C6 and C7 (for example, from different ring current shielding effects).

3.2. E. coli *DHFR–MTX–NADPH and DHFR–MTX–NADP⁺ complexes*

The ternary enzyme complex formed with [2,4a,6-^{13}C]MTX and NADPH at pH 6.5 and 7.2 shows only one set of ^{13}C signals for the bound MTX (see Table I and Fig. 2b), indicating that only one form of this ternary complex can be detected. This agrees with the findings of Falzone et al. [6] who showed that the ^1H spectrum of the DHFR–NADPH–MTX complex has no doubling of signals and points to a single conformational form.

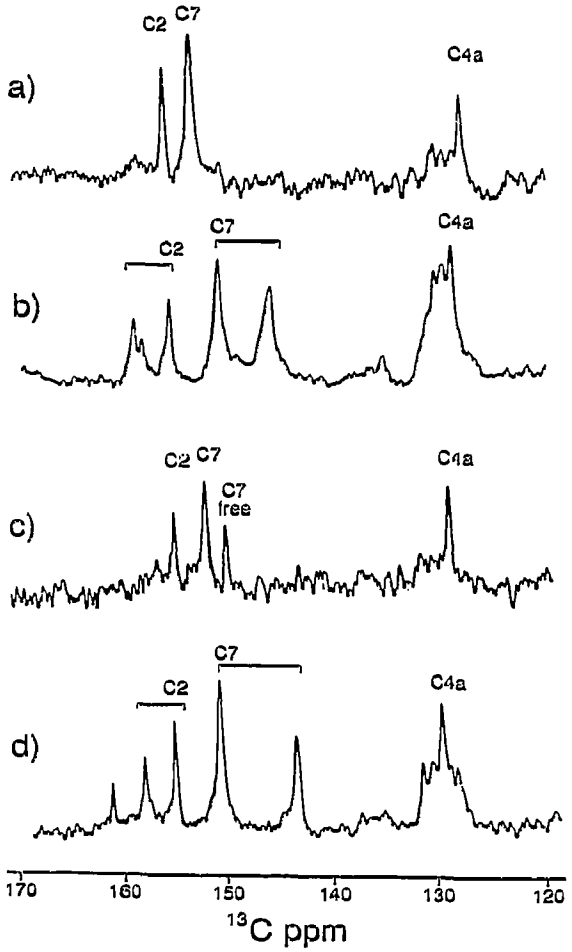


Fig. 3. ^{13}C NMR spectra of complexes formed with [2,4a,7,9-^{13}C]folic acid and (a) *E. coli* DHFR (pH 7.5), (b) *L. casei* DHFR (pH 7.0), (c) *E. coli* DHFR and $NADP^+$ (pH 7.4) and (d) *L. casei* DHFR and $NADP^+$ (pH 7.3). Spectra were obtained at 298 K and 100 MHz (C9 region not shown).

The ^{13}C spectra of the ternary complexes formed with $NADP^+$ and the two labelled MTXs at pH 4.8–7.2 do not show any resolved splittings for the carbons of the bound MTXs (see Fig. 2c), but the signals are broader than those observed in the ternary complex with NADPH. These line broadenings do not change with temperature and are thus unrelated to exchange processes.

3.3. E. coli *DHFR–folate complex*

The ^{13}C spectra of the *E. coli* DHFR binary complexes with [4,6,8a-^{13}C] and [2,4a,7,9-^{13}C]folate have been examined. In each case only a single set of ^{13}C signals for bound folate was observed (Table I, see Fig. 3a). This indicates the presence of a single conformation

in these complexes in solution, in agreement with the recent ^{1}H and ^{15}N NMR studies of Falzone et al. [11] and Huang et al. [7]. The ^{13}C chemical shifts of bound folate do not vary over the pH range 5.0–7.5. It is interesting that folate, which binds less tightly to DHFR than does MTX, selects out a single conformational form upon complexing with the *E. coli* enzyme, whereas MTX binds in two forms with comparable binding energy (see above). This behaviour contrasts with that which we observed earlier for the corresponding complexes formed with the *L. casei* enzyme where folate binds in at least two forms (see multiple bound signals in Fig. 3b) [13,21], while MTX binds in a single conformation [12,13].

3.4. E. coli *DHFR–folate–NADP$^+$ complex*

We have also studied the ^{13}C spectrum of the *E. coli* DHFR complex with $NADP^+$ and with either [4,6,8a-^{13}C]- or [2,4a,7,9-^{13}C]folate. In each case a single set of ^{13}C signals for bound folate was observed (Table I, Fig. 3c). A single ^{15}N signal was earlier observed for the corresponding complex with [5-^{15}N]folate [7]. Thus, in solution folate binds in the presence of $NADP^+$ to *E. coli* DHFR in a single conformational form.

The ^{13}C chemical shifts of bound folate in the $NADP^+$ ternary complex with *E. coli* DHFR do not vary over the pH range 5.4–7.5, and are similar to those of the binary complex (Table I). These ^{13}C chemical shifts of bound folate also resemble those measured for free folic acid at pH 5.5 (Table I), which is known to exist in the 4-keto form and with N1 not protonated [20]. It is likely that a similar structure is adopted by the pteridine ring in bound folate.

In our earlier work on folate binding to *L. casei* DHFR, the existence of multiple forms of the ternary $NADP^+$ complex was demonstrated; two of the forms have 180° differences in pteridine ring orientations, as shown by NOE experiments [4,13]. The ^{13}C chemical shifts of the pteridine ring in one of these forms, Form IIb, are similar to those measured in the present work for the corresponding *E. coli* DHFR complex (Table I, see Fig. 3d, vs. c), suggesting a similarity in pteridine structure and orientation. In contrast, other forms of the ternary *L. casei* DHFR complex give rise to ^{13}C chemical shifts which are very different (data not given here) reflecting differences in pteridine structure and orientation [13].

The inter-species differences in the ability to form multiple conformations, as shown by DHFR complexes with inhibitors and with substrates will be further explored using other ^{13}C-enriched ligands.

Acknowledgements: We are grateful to J.F. Morrison for a generous gift of the *E. coli* DHFR and to G. Ostler, J. McCormick and D.D. Chau for expert technical assistance. The NMR measurements were made using the facilities at the MRC Biomedical NMR Centre, National Institute for Medical Research, Mill Hill. HTAC acknowledges funding support by the Australian Research Council, and the award of a travel grant from the Wellcome Trust and the Clive and Vera Ramaciotti Foundations.

REFERENCES

[1] Blakley, R.L. (1985) in: Folates and Pterins, vol. 1, (R.L. Blakley and S.J. Benkovic, eds.) pp. 191–253, Wiley, New York.
[2] Gronenborn, A., Birdsall, B., Hude, E., Roberts, G.C.K., Feeney, J. and Burgen, A.S.V. (1981) Mol. Pharmacol. 20, 145–153.
[3] Cheung, H.T.A., Searle, M.S., Feeney, J., Birdsall, B., Roberts, G.C.K., Kompis, I. and Hammond, S.J. (1986) Biochemistry 25, 1925–1931.
[4] Birdsall, B., Feeney, J., Tendler, S.J.B., Hammond, S.J. and Roberts, G.C.K. (1989) Biochemistry 28, 2297–2305.
[5] Birdsall, B., Tendler, S.J.B., Arnold, J.R.P., Feeney, J., Griffin, R.J., Carr, M.D., Thomas, J.A., Roberts, G.C.K. and Stevens, M.F.G. (1990) Biochemistry 29, 9660–9667.
[6] Falzone, C.J., Wright, P.E. and Benkovic, S.J. (1991) Biochemistry 30, 2184–2191.
[7] Huang, F.-Y., Yang, Q.-X. and Huang, T.-H. (1991) FEBS Lett. 289, 231–234.
[8] Huang, F.-Y., Yang, Q.-X., Huang, T.-H., Gelbaum, L. and Kuyper, L.F. (1991) FEBS Lett. 283, 44–46.
[9] Groff, J.P., London, R.E., Cocco, L. and Blakley, R.L. (1981) Biochemistry 20, 6169–6178.
[10] Feeney, J. (1990) Biochem. Pharmacol. 40, 141–152.
[11] Falzone, C.J., Benkovic, S.J. and Wright, P.E. (1990) Biochemistry 29, 9667–9677.
[12] Birdsall, B., Andrews, J., Ostler, G., Tendler, S.J.B., Feeney, J., Roberts, G.C.K., Davies, R.W. and Cheung, H.T.A. (1989) Biochemistry 28, 1353–1362.
[13] Cheung, H.T.A., Birdsall, B., Frenkiel, T.A., Chau, D.D. and Feeney, J. (1992) manuscript in preparation.
[14] Cheung, H.T.A., Smal, M. and Chau, D.D. (1987) Heterocycles 25, 507–514.
[15] Rood, J.I. and Williams, J.W. (1981) Biochim. Biophys. Acta 660, 214–218.
[16] Levitt, M.H., Freeman, R. and Frenkiel, T.A. (1983) Adv. Magn. Reson. 11, 47–110.
[17] Gronenborn, A.M., Bax, A., Wingfield, P.T. and Clore, G.M. (1989) FEBS Lett. 243, 93–98.
[18] Cocco, L., Temple Jr. C., Montgomery, J.A., London, R.E. and Blakley, R.L. (1981) Biochem. Biophys. Res. Commun. 100, 413–419.
[19] Bolin, J.T., Filman, D.J., Matthews, D.A. and Kraut, J. (1982) J. Biol. Chem. 257, 13650–13662.
[20] Blakley, R.L. (1969) The Biochemistry of Folic Acid and Related Pteridines, Elsevier, Amsterdam.
[21] Birdsall, B., DeGraw, J., Feeney, J., Hammond, S.J., Searle, M.S., Roberts, G.C.K., Colwell, W.T. and Crase, J. (1987) FEBS Lett. 217, 106–110.