THE CHARGE STATE OF TRIMETHOPRIM BOUND TO *LACTOBACILLUS CASEI* DIHYDROFOLATE REDUCTASE

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Received 24 June 1981

1. Introduction

Dihydrofolate reductase is the 'target' for the antifolate drugs, which include the antineoplastic agent methotrexate and the antibacterial agent trimethoprim. Methotrexate, a 2,4-diaminopteridine, is a close structural analogue of the substrate folate, a 2-amino-4-oxopteridine, but binds about a thousand times more tightly [1–3]. Trimethoprim is a 2,4-diaminopyrimidine, and compounds of this type also bind much more tightly than their 4-oxo analogues (e.g. [4]).

There appear to be two major differences between the binding of methotrexate and that of folate: methotrexate is protonated at N1 when bound to the enzyme [2–10], while folate is apparently not, and the pteridine ring of methotrexate binds to the enzyme in an orientation which differs by a rotation of about 180° about its long axis from that adopted by folate [11–14]. The recent determination of the crystal structure of the enzyme–trimethoprim complex [15] shows that the orientation of the 2,4-diaminopyrimidine ring in the binding site is very similar to that of the analogous part of methotrexate [11,16]. We now report ^{13}C NMR experiments which confirm this similarity in the binding of the two inhibitors in solution, and show that trimethoprim is also protonated when bound.

2. Materials and methods

Dihydrofolate reductase was isolated and purified from *Lactobacillus casei* MTX/R as in [17]. Trimethoprim enriched to 90% with ^{13}C at the 2-position of the pyrimidine ring was prepared by condensation of [^{13}C]guanidine with β-anilino-α-(3,4,5-trimethoxybenzyl) acrylonitrite [24,25].

^{13}C NMR spectra were obtained at 50.3 MHz using a Bruker WM-200 spectrometer. Samples contained 0.5 mM enzyme and 0.4–1.0 mM [2-^{13}C]trimethoprim in ^2H_2O containing 50 mM phosphate and 500 mM KCl, with a total volume of 3 ml. The pH* (a meter reading uncorrected for the isotope effect on the glass electrode) was adjusted with small volumes of 1 M ^2HCl or KO^2H. Spectra were acquired at a sample temperature of 11°C using a spectral width of 10 kHz and 16384 data points (final digital resolution 1.2 Hz/point); the pulse interval was 0.82 s, and the flip angle 25°. Up to 60 000 transients were averaged, and before Fourier transformation the free induction decay was multiplied by an exponential function to improve the signal-to-noise ratio, leading to line-broadening of 2–5 Hz. Chemical shifts are given with respect to external dioxan.

3. Results and discussion

As shown in fig.1, when [2-^{13}C]trimethoprim is added to *L. casei* dihydrofolate reductase at a molar ratio to the enzyme of 2:1, two separate resonances are seen for the ^{13}C-enriched 2-carbon. At a molar ratio of 0.9:1, only the resonance at 89.26 ppm is observed, thus identifying this as the resonance of the bound trimethoprim and indicating that exchange of trimethoprim between bound and free states is slow on the NMR time-scale at 11°C. (This is to be expected, since the rate constant for dissociation of trimethoprim from the complex is only 6 s^{-1} at 45°C [18].)

When the pH* of the sample is increased from 6.53 to 7.46, the chemical shift of the resonance of bound trimethoprim is unaffected, while that of free

Published by Elsevier/North-Holland Biomedical Press
00145793/81/0000–0000/$02.50

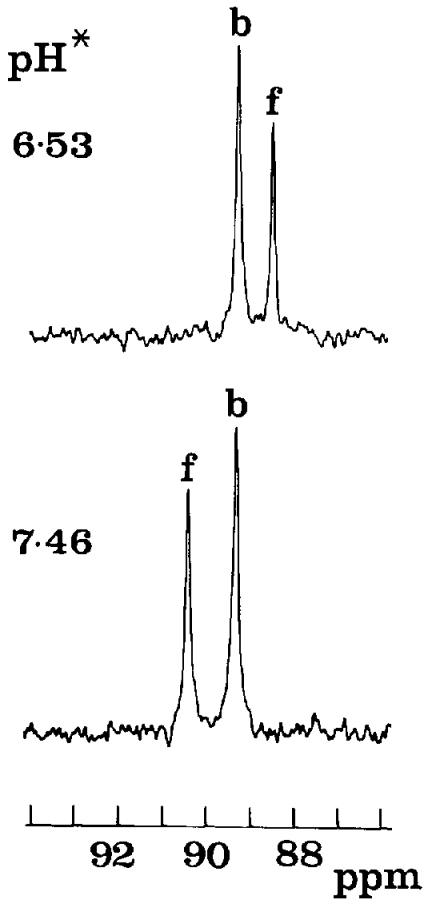

Fig.1. ^{13}C Resonances, measured at 50.3 MHz, of [2-^{13}C]trimethoprim (1 mM) in the presence of *L. casei* dihydrofolate reductase (0.5 mM) at two pH* values. The resonances from trimethoprim bound to the enzyme and free in solution are labelled b and f, respectively.

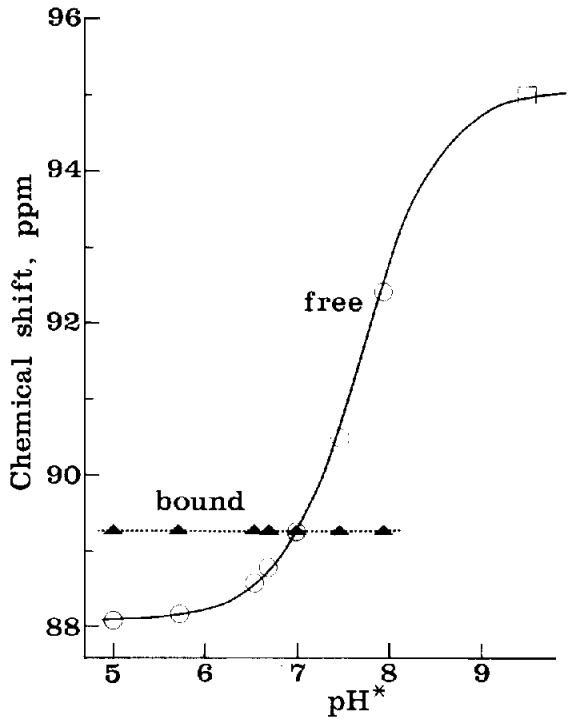

Fig.2. The pH*-dependence of the chemical shift of the 2-^{13}C resonance of trimethoprim free (○) and bound to *L. casei* dihydrofolate reductase (▲). Data between pH* 5 and 8 were obtained from a sample containing dihydrofolate reductase and 2 molar equivalents of [2-^{13}C]trimethoprim. The enzyme is unstable above pH* 8, and the data point for free trimethoprim at pH* 9.5 was obtained in the absence of enzyme. The solid line is calculated for pK 7.70 and a chemical shift difference between protonated and unprotonated forms of 7.09 ppm.

trimethoprim shifts downfield. The pH*-dependence of the chemical shifts of these two signals is shown in more detail in fig.2. The resonance of free trimethoprim shows a marked downfield shift with increasing pH* as the molecule deprotonates; the total titration shift, measured in the absence of enzyme, is 7.09 ppm, from 87.97 ppm to 95.06 ppm with an apparent pK_a (in ^2H_2O containing 0.5 M KCl, and based on pH* measurements) of 7.7. By contrast, the chemical shift of the resonance of bound trimethoprim remains constant over pH* 5–8.

The chemical shift of the 2-carbon of bound trimethoprim is clearly much closer to that of the protonated than to that of the unprotonated molecule. The difference of 1.29 ppm between the chemical shift of the bound and the protonated molecules can, as discussed below, readily be accounted for by the kind of environmental effects expected for a bound ligand, whereas the difference of 5.80 ppm between the shifts of the bound and the unprotonated molecules is outside the range expected for such effects in diamagnetic, non-haem proteins. It is therefore highly probable that, like methotrexate, trimethoprim is protonated when bound to dihydrofolate reductase. The pH*-dependence of the chemical shift of the 2-carbon resonance of the bound ligand up to pH* 8 shows that there is no detectable deprotonation of the ligand over this range, and hence that its pK must have increased by at least 2 units on binding.

We have earlier used UV absorption spectroscopy in an attempt to determine the charge state of enzyme-bound methotrexate and trimethoprim [2]. In these

experiments, a pH-dependence of the UV difference spectrum was observed which was ascribed to deprotonation of the enzyme-bound ligand, and it was concluded that the pK of trimethoprim was increased by only ~0.8 units on binding. The present ^{13}C NMR experiments, which provide much more direct information on the charge state of the molecule, show that the increase in pK must be much greater than this. Similarly, ^{13}C NMR experiments on the binding of [2-^{13}C]methotrexate of the *L. casei* reductase [3] showed that the UV experiments also led to an underestimate of the increase in pK of this ligand on binding; both methotrexate and trimethoprim have pK values >9.5–10 when bound to the reductase. The pH-dependence of the UV spectrum must arise from the ionisation of some group or groups on the enzyme.

The substantial increase in pK of trimethoprim on binding makes a major contribution to the observed change in 2-^{13}C chemical shift on binding. However, this is not the sole component of the change in chemical shift, since, as noted above, the resonance of trimethoprim in the bound state is 1.29 ppm downfield of that of free protonated trimethoprim. This second component of the chemical shift change obviously reflects the environment of the protonated trimethoprim in the binding site. From our previous calculations [18], any ring-current contributions (from Phe-30 or the benzyl ring of trimethoprim itself) are likely to be small. Two other probable contributions can be identified. In the crystal, the carboxylate of Asp-26 (*L. casei* numbering) is close to N1 of bound methotrexate [11,16] or trimethoprim [15]. This interaction is doubtless responsible for the increase in pK of these ligands on binding, but the proximity of the carboxylate group will also lead to an electric field shift of the 2-^{13}C resonance, whose sign will depend on the relative polarisability of the bonds to this carbon [19–21]. In addition, the 2-amino group probably forms a hydrogen-bond to Thr 116 (*L. casei* numbering) [11,15,16] and this will lead to a shift of the resonance of the directly bonded carbon. For example, in aniline the C_α resonance is 2.1–2.7 ppm further downfield in dimethylsulphoxide or acetone solution than in carbon tetrachloride solution, presumably due, at least in part, to hydrogen-bonding of the amino group [22].

Whatever the precise origin of this second component of the change in chemical shift on binding, it is notable that it is the same for trimethoprim (1.29 ppm) as for methotrexate (1.32 ppm; [3]). This must imply that the environment of the 2-carbon, and hence probably that of the amidinium moiety (N1, C2, N3 and the 2-amino group) as a whole, is identical in the enzyme–trimethoprim and enzyme–methotrexate complexes in solution. In view of the considerable sensitivity of ^{13}C chemical shifts to environment and electron distribution (e.g. [23]) this statement can be made with some confidence; it is clearly consistent with recent crystallographic work [15]. This close similarity in the binding of the corresponding parts of trimethoprim and methotrexate justifies one of the central assumptions of our analysis of the proton chemical shift changes of trimethoprim on binding [18], and indeed one of the two possible conformations for trimethoprim bound to the enzyme in solution proposed from this analysis [18] is very similar to that subsequently found crystallographically [15].

Acknowledgements

We are most grateful to Professor R. L. Blakley for his generous gift of [^{13}C]guanidine and for information on his experiments prior to publication, and to Gill Ostler and John McCormick for invaluable technical assistance.

References

[1] Blakley, R. L. (1969) The biochemistry of folic acid and related pteridines, Elsevier/North-Holland, Amsterdam, New York.
[2] Hood, K. and Roberts, G. C. K. (1978) Biochem. J. 171, 357.
[3] Cocco, L., Temple, C. jr, Montgomery, J. A., London, R. E. and Blakley, R. L. (1981) Biochem. Biophys. Res. Commun. in press.
[4] Baker, B. R. (1967) Design of active-site-directed irreversible enzyme inhibitors, Wiley, New York.
[5] Erickson, J. S. and Mathews, C. K. (1972) J. Biol. Chem. 247, 5661.
[6] Poe, M., Greenfield, N. J., Hirshfield, J. M. and Hoogsteen, K. (1974) Cancer Biochem. Biophys. 1, 7.
[7] Gupta, S. V., Greenfield, N. J., Poe, M., Makulu, D. R., Williams, M. N., Moronson, B. A. and Bertino, J. R. (1977) Biochemistry 16, 3073.
[8] Saperstein, D. D., Rein, A. J., Poe, M. and Leahy, M. F. (1978) J. Am. Chem. Soc. 100, 4296.
[9] Ozaki, Y., King, R. W. and Carey, P. R. (1981) Biochemistry 20, 3219.
[10] Cocco, L., Groff, J. P., Temple, C. jr, Montgomery, J. A., London, R. E., Matwiyoff, N. A. and Blakley, R. L. (1981) Biochemistry in press.

[11] Matthews, D. A., Alden, R. A., Bolin, J. T., Filman, D. J., Freer, S. T., Hamlin, R., Hol, W. G. J., Kisliuk, R. L., Patore, E. J., Plante, L. T., Xuong, N. and Kraut, J. (1978) J. Biol. Chem. 253, 6946.
[12] Fonticella-Camps, J. C., Bugg, C. E., Temple, C. jr, Rose, J. D., Montgomery, J. A. and Kisliuk, R. L. (1979) J. Am. Chem. Soc. 101, 6114.
[13] Hitchings, G. H. and Roth, B. (1980) in: Enzyme inhibitors as drugs, (Sandler, M. ed) Macmillan, London.
[14] Charlton, P. A., Young, D. W., Birdsall, B., Feeney, J. and Roberts, G. C. K. (1979) J. Chem. Soc. Chem. Commun. 922.
[15] Baker, D. J., Beddell, C. R., Champness, J. N., Goodford, P. J., Norrington, F. E. A., Smith, D. R. and Stammers, D. K. (1981) FEBS Lett. 126, 49.
[16] Matthews, D. A., Alden, R. A., Bolin, J. T., Freer, S. T., Hamlin, R., Xuong, N., Kraut, J., Poe, M., Williams, M. N. and Hoogsteen, K. (1977) Science 197, 452.
[17] Dann, J. G., Ostler, G., Bjur, R. A., King, R. W., Scudder, P., Turner, P. C., Roberts, G. C. K., Burgen, A. S. V. and Harding, N. G. L. (1976) Biochem. J. 157, 559.
[18] Cayley, P. J., Albrand, J. P., Feeney, J., Roberts, G. C. K., Piper, E. A. and Burgen, A. S. V. (1979) Biochemistry 18, 3886.
[19] Buckingham, A. D., Bernstein, H. J. and Raynes, W. J. (1962) J. Chem. Phys. 36, 3481.
[20] Batchelor, J. G., (1975) J. Am. Chem. Soc. 97, 3410.
[21] Batchelor, J. G., Feeney, J. and Roberts, G. C. K. (1975) J. Magn. Res. 20, 19.
[22] Nelson, G. L., Levy, G. C. and Cargioli, J. D. (1972) J. Am. Chem. Soc. 94, 3090.
[23] Levy, G. C., Lichter, R. L. and Nelson, G. L. (1980) Carbon-13 nuclear magnetic resonance spectroscopy, 2nd edn, Wiley, New York.
[24] Cresswell, R. M. and Mentha, J. W. (1975) US patent 3, 878, 252.
[25] Cresswell, R. M., Mentha, J. W. and Seaman, R. L. (1972) US patent 3, 697, 512.