Vol. 101, No. 4, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1981 Pages 1139-1144

^{13}C NMR EVIDENCE FOR THREE SLOWLY INTERCONVERTING CONFORMATIONS OF THE DIHYDROFOLATE REDUCTASE-NADP$^+$-FOLATE COMPLEX

B. Birdsall, A. Gronenborn, G.M. Clore, G.C.K. Roberts*, J. Feeney and A.S.V. Burgen

Division of Molecular Pharmacology, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

Received June 24, 1981

SUMMARY

The 3-carboxamido-^{13}C resonance of NADP$^+$ in its complex with Lactobacillus casei dihydrofolate reductase and folate has been studied as a function of pH. At low pH a single resonance is observed, while at high pH two resonances are observed, neither of which has the same chemical shift as that seen at low pH. The rates of interconversion between the three states of the complex represented by these resonances are $<19\ s^{-1}$ at 11°C.

INTRODUCTION

One of the most useful contributions of nmr spectroscopy to our understanding of proteins is in the study of dynamic aspects of their behaviour in solution, particularly in the characterisation of interconverting conformational states which may not be detectable in the crystal.

The importance of dihydrofolate reductase as the 'target' for the antifolate drugs, including methotrexate and trimethoprim, has led to detailed studies of this enzyme by X-ray crystallography (1,2,3) and nmr spectroscopy (e.g., 4-12). It has been found that dihydrofolate reductase can exist in solution in at least two slowly interconverting conformational states, both in the absence of ligands (4,13,14) and in the enzyme-trimethoprim-NADP$^+$ ternary complex (5,6). The existence of these conformational states has important implications for understanding the specificity of the enzyme, since they differ in their affinity for various substrate analogues.

* To whom correspondence should be addressed.

0006-291X/81/161139-06$01.00/0

We now report that an enzyme-substrate complex, the reductase-$NADP^+$-folate complex, exists in three distinguishable states whose proportions vary with pH. This complex is of interest both as a dead-end complex formed during the reduction of folate and for comparison with the analogous complex formed with the inhibitor methotrexate.

MATERIALS AND METHODS

Dihydrofolate reductase was isolated and purified from Lactobacillus casei MTX/R as described earlier (15). Its concentration was determined by measuring its catalytic activity and by methotrexate titration (15). $NADP^+$ enriched to 90% with ^{13}C in the carboxamido group on the nicotinamide ring was prepared from $^{13}CO_2$ as described by Way et al. (7). Samples for nmr spectroscopy contained 0.5 mM enzyme in ^2H_2O containing 500 mM KCl, 50 mM potassium phosphate, initially at pH* (uncorrected meter reading) 6.5. The pH was adjusted using microlitre volumes of 0.1 or 1 M HCl or KOH, and measured at the temperature of the nmr experiment.

^{13}C nmr spectra were obtained at 50.3 MHz using a Bruker WM200 spectrometer. A spectral width of 10 KHz and a data table of 16384 points were used, giving a pulse interval of 0.819 s and digital resolution of 1.2 Hz/point. The flip angle was 25°; typically 50,000 to 80,000 transients were averaged. Before Fourier transformation, the free induction decay was multiplied by an exponential function giving a linebroadening of 5 Hz. Chemical shifts are expressed relative to dioxan, with downfield shifts positive.

RESULTS AND DISCUSSION

The carboxamido ^{13}C resonance of [3-carboxamido-^{13}C]$NADP^+$ in the L.casei dihydrofolate reductase-$NADP^+$-folate complex is shown in Figure 1 at three pH* values. The sample contained a molar ratio of $NADP^+$ to enzyme of 0.9, and with a binding constant of 1.2×10^6 M^{-1} (16) essentially all the $NADP^+$ is bound to the enzyme; the resonance of free $NADP^+$ would appear at 98.58 ppm.

At pH*5.5, a single major resonance from the bound $NADP^+$ is observed, at 96.32 ppm, labelled a in Figure 1. As the pH* is increased, the intensity of resonance a decreases, and that of two other resonances, labelled b and c, increases. By pH* 7.33, resonances b and c at 94.26 and 95.88 ppm predominate and resonance a is barely detectable. The chemical shifts of resonances a, b and c are each independent of pH*; only their relative intensities vary, and these are summarised in Table 1. In our earlier studies of [3-carboxamido-^{13}C]$NADP^+$ binding to the enzyme (7) at 25.2 MHz and pH* 6.5, two resonances were observed for the enzyme-$NADP^+$-folate complex, 2.6 and 4.33 ppm upfield

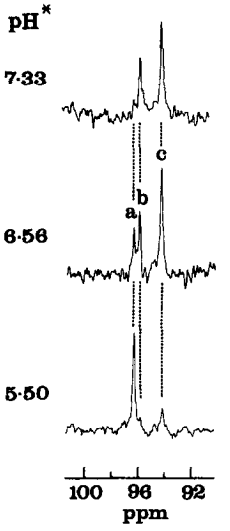

Figure 1. Carboxamide carbon region of the 50.3 MHz ^{13}C spectrum of the complex of dihydrofolate reductase with folate and [3-carboxamido-^{13}C]$NADP^+$ at the pH* values indicated. (pH* denotes a meter reading uncorrected for the deuterium isotope effect on the glass electrode.)

from free $NADP^+$ (96.0 and 94.25 ppm from dioxan); resonances a and b were barely resolved at the lower field.

The observation that the relative intensities but not the chemical shifts of the three resonances are pH-dependent shows that the three states of the complex which they represent interconvert slowly on the nmr timescale. Resonances a and b are separated by only 22 Hz at 50.3 MHz, so the observation of clearly resolved resonances (e.g. at pH* 6.56, Fig.1) shows

Table 1. Relative intensities of carboxamide ^{13}C resonances a, b and c of the enzyme-$NADP^+$-folate complex

pH*	Fractional intensity			Intensity ratio	
	a (96.32)†	b (95.88)†	c (94.26)†	a/c	c/b
5.50	0.73	0.1	0.17	4.29	1.7
6.05	0.49	0.19	0.32	1.53	1.68
6.56	0.22	0.29	0.49	0.45	1.69
7.33	0.1	0.34	0.56	0.18	1.65

† Chemical shift, ppm from dioxan

that the rate of interconversion is less than 35 s^{-1}. The linewidths of the three resonances at 11°C average 6 Hz (after subtracting the contribution from the exponential function used to improve the signal-to-noise ratio), which sets an upper limit to all the interconversion rates of 19 s^{-1}.

Provided that the spin-lattice relaxation rate of the carboxamido carbon is similar in the three states of the complex, the relative intensities of resonances a, b and c given in Table 1 can be used to calculate the equilibrium constants between these states. As can be seen from Table 1, the ratio of the intensities of resonances c and b is pH-independent, with a value of 1.67 (± 0.02). The equilibrium constant between states a and c (from the intensities of resonances a and c) is, however, markedly pH-dependent. Since the interconversion rate is so slow, it is unlikely that the only difference between state a on the one hand and states b and c on the other is the state of protonation of one or more groups in the complex. It is much more probable that the ionisation is coupled to a (slow) conformational change which is responsible for the observed change in environment of the carboxamido carbon of $NADP^+$. The pH-dependence of the fractional intensity of resonance a can be adequately described by a model in which a single group in the complex has a pK $\geqslant$ 7.0 in state a, but a pK $\leqslant$ 5.0 in states b and c (since the equilibrium constant c/b is pH-independent over the range 5-7, this group must have the same pK in both these states). The possible identity of this group will be discussed elsewhere (17).

Protonation of this group thus favours a single state of the complex, as far as the carboxamido carbon is concerned, while when the group is unprotonated the complex exists as an equilibrium mixture of two states, in one of which the environment of the carboxamido carbon is similar but not identical to that at low pH (state a). The shifts of resonances a and b (96.32 and 95.88 ppm) are similar to those of the single resonances seen for this $NADP^+$ ^{13}C resonance in the ternary complexes with the inhibitors methotrexate and aminopterin (95.96 and 95.68 ppm, respectively; ref. 7),

0.6 - 1.3 ppm upfield of the resonance position in the binary enzyme-$NADP^+$ complex. Resonance c, howevever, 1.62 ppm upfield of resonance b, is at much higher field than the carboxamido ^{13}C resonance in any other complex; in this conformational state, the environment of the carboxamido group appears to be unique to the folate complex. The nature of the structural differences between conformations b and c which give rise to this striking difference in ^{13}C chemical shift remain to be established. ^{1}H and ^{31}P nmr studies, to be described elsewhere (17), and the studies of [benzoyl carbonyl-^{13}C]folate by Pastore et al. (18), indicate that the differences involve only the immediate vicinity of the nicotinamide ring, and are quite distinct from those described for the two conformations of the enzyme-$NADP^+$-trimethoprim complex (6). The observation of another distinct set of slowly interconverting conformational states is further evidence of the remarkable conformational adaptability of this enzyme.

ACKNOWLEDGEMENTS

We are most grateful to Dr. J.L. Way for the synthesis of [carboxamido-^{13}C]$NADP^+$, and to Gill Ostler and John McCormick for invaluable technical assistance.

REFERENCES

1. Matthews, D.A., Alden, R.A., Bolin, J.T., Filman, D.J., Freer, S.T., Hamlin, R., Hol, W.G.J., Kisliuk, R.L., Pastore, E.J., Plante, L.T., Xuong, N., and Kraut, J. (1978) J. Biol. Chem., 253, 6946-6954.
2. Matthews, D.A., Alden, R.A., Freer, S.T., Xuong, N., and Kraut, J. (1979) J. Biol. Chem., 254, 4144-4151.
3. 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, 49-52.
4. London, R.E., Groff, J.P., and Blakley, R.L. (1979) Biochem. Biophys. Res. Comm., 86, 779-786.
5. Gronenborn, A., Birdsall, B., Hyde, E.I., Roberts, G.C.K., Feeney, J., and Burgen, A.S.V. (1981) Nature, 290, 273-274.
6. Gronenborn, A., Birdsall, B., Hyde, E.I., Roberts, G.C.K., Feeney, J., and Burgen, A.S.V. (1981) Mol. Pharmacol., in press.
7. Way, J.L., Birdsall, B., Feeney, J., Roberts, G.C.K., and Burgen, A.S.V. (1975) Biochemistry, 14, 3470-3475.
8. Feeney, J., Birdsall, B., Roberts, G.C.K., and Burgen, A.S.V. (1975) Nature, 257, 564-566.
9. Feeney, J., Roberts, G.C.K., Birdsall, B., Griffiths, D.V., King, R.W., Scudder, P., and Burgen, A.S.V. (1977) Proc. R. Soc.Lond., ser. B., 196, 267-290.

10. Hyde, E.I., Birdsall, B., Roberts, G.C.K., Feeney, J., and Burgen, A.S.V. (1980) Biochemistry, 19, 3728-3746.
11. Cocco, L., Blakley, R.L., Walker, T.E., London, R.E., and Matwiyoff, N.A. (1978) Biochemistry, 17, 4285-4290.
12. Blakley, R.L., Cocco, L., London, R.E., Walker, T.E., and Matwiyoff, N.A. (1978) Biochemistry, 17, 2284-2292.
13. Dunn, S.M.J., Batchelor, J.G., and King, R.W. (1978) Biochemistry, 17, 2356-2364.
14. Pattishall, K.H., Burchall, J.J., and Harvey, R.J. (1976) J. Biol. Chem., 251, 7011-7020.
15. 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, 559-571.
16. Birdsall, B., Burgen, A.S.V., and Roberts, G.C.K. (1980), Biochemistry, 19, 3723-3731.
17. Birdsall, B., Gronenborn, A., Clore, G.M., Hyde, E.I., Roberts, G.C.K., Feeney, J., and Burgen, A.S.V., manuscript in preparation.
18. Pastore, E.J., Plante, L.T., Wright, J.M., Kisliuk, R.L., and Kaplan, N.O. (1976) Biochem. Biophys. Res. Comm., 68, 471-475.