R67 is a Type II dihydrofolate reductase (DHFR) that catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate by facilitating the addition of a proton to N5 of DHF and the transfer of a hydride ion from NADPH to C6. Because this enzyme is a plasmid-encoded DHFR from trimethoprim-resistant bacteria, extensive studies on R67 with various methods have been performed to elucidate its reaction mechanism. Here, Raman difference measurements, conducted on the ternary complex of R67-NADP⁺-DHF believed to be an accurate mimic of the productive DHFR/NADPH-DHF complex, show that the $pK_a$ of N5 in the complex is less than 4. This is in clear contrast to the behavior observed in Escherichia coli DHFR, a substantially more efficient enzyme, where the $pK_a$ of bound DHF at N5 is increased to 6.5 compared with its solution value of 2.6. A comparison of the ternary complexes in R67 and E. coli DHFRs suggests that enzymic raising of the $pK_a$ at N5 can significantly increase the catalytic efficiency of the hydride transfer step. However, R67 shows that even without such a strategy an effective DHFR can still be designed.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP oxidoreductase, EC 1.5.1.3, DHFR) catalyzes the reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF) by facilitating the addition of a proton to N5 of DHF and the transfer of a hydride ion from NADPH to C6 (Scheme I). DHFR is an important enzyme as it is required for the production of purines, thymidylate, and a few amino acids; therefore, it has been the target of antimicrobial drugs for a long time. Various drug-resistant types of DHFR have been discovered, and it has been found that Type II DHFRs are particularly interesting as they are genetically unrelated to chromosomal DHFRs. One Type II DHFR, R67, is a homotetramer, each monomer consisting of 78 amino acids. Crystallographic studies of R67 show a very different structure compared with chromosomal DHFR, either at the level of the overall protein fold or at the active site (1, 2). Yet R67 compares quite well as an enzyme; its $k_{cat} = 1.3 \text{ s}^{-1}$ (3) can be compared with a hydride transfer rate of 238 $\text{s}^{-1}$ for chromosomal DHFR at pH 7 (4, 5). Hence, it is interesting to compare the catalytic mechanism of R67 with chromosomal DHFRs (2).

Extensive kinetic, site-directed mutagenesis, x-ray crystallographic, and theoretical molecular modeling studies have been performed on Escherichia coli chromosomal DHFR to elucidate its reaction mechanism (6–9). The electronic nature of the ground state within the active site in the productive DHFR-NADP-DHF complex is unclear, and this is important to an understanding of the reaction mechanism of DHFR. A key issue has to do with whether or not N5 of the pteridine ring of DHF is protonated in the ground state in the DHFR-NADPH-DHF complex and hence precedes hydride transfer or occurs later in the reaction pathway. A recent study using Raman difference spectroscopy of the E. coli DHFR-NADP⁺-DHF complex, believed to be an accurate mimic of the productive DHFR/NADPH-DHF complex, identified two N5=C6 stretch “marker” bands indicating either an unprotonated (1650 cm⁻¹) or protonated state (1675 cm⁻¹) for N5 (10, 11). A titration study, using the 1650 and 1675 cm⁻¹ marker bands, showed that the $pK_a$ of N5 is raised from 2.6 in solution (12) to 6.5 in this complex (10). Thus, there is good reason to believe that, at least for DHFR from E. coli, N5 is likely protonated in the ground state of the DHFR-NADPH-DHF complex. Moreover, this conclusion agreed well with pH-dependent kinetic and mutagenesis studies of the E. coli enzyme (4, 13, 14). A protein-induced “preprotonation” of N5 goes a long way toward understanding the catalytic mechanism because N5 must protonate along the pathway and also ab initio calculations suggest that protonation will facilitate hydride transfer (10, 15, 16). On the other hand, difference Raman studies of dihydrooropterin aldolase, another enzyme in the folate synthesis pathway whose structural and chemical similarities to E. coli DHFR prompted the suggestion of a reaction mechanism similar to that of chromosomal DHFRs (17), found that N5 is unprotonated above pH 6 (18). Therefore, the reaction mechanism involving hydride transfer to the C6 carbon of the dihydropterin ring catalyzed by DHFR may be different depending on the source.

Thus, toward the goal of determining if Type II R67 DHFR adopts a reaction mechanism similar to that of E. coli DHFR, we have carried out studies on R67 under various conditions by Raman difference spectroscopy (19). The principal aim of this study is to determine the $pK_a$ of N5 for DHF bound to R67. Our vibrational spectral results of DHF bound to R67 also report on small changes in the distribution of electrons within bonds of DHF that come about upon binding. Significant frequency changes are observed for the C6=N5 stretch frequency for bound DHF compared with the solution counterpart. However, N5 of DHF in various protein complexes is not protonated at pH 5 and above. The significance of this finding will be discussed.

This paper is available on line at http://www.jbc.org

Hua Deng‡ § Robert Callender‡ and Elizabeth Howell¶

From the ‡Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461 and ¶Department of Biochemistry, Cell and Molecular Biology, University of Tennessee, Knoxville, Tennessee 37966

© 2001 by The American Society for Biochemistry and Molecular Biology, Inc.

Published, JBC Papers in Press, October 25, 2001, DOI 10.1074/jbc.M105107200

This journal and the individual contributors grant to The Journals of The American Society for Biochemistry and Molecular Biology the royalty free, irrevocable, worldwide license to publish this article, to represent this article in derivative works, to disseminate and make available the article to the public for the purposes of education and research, and to permit others to do so.

Received for publication, June 4, 2001, and in revised form, October 24, 2001
Published, JBC Papers in Press, October 25, 2001, DOI 10.1074/jbc.M105107200

This paper is available on line at http://www.jbc.org

This journal and the individual contributors grant to The Journals of The American Society for Biochemistry and Molecular Biology the royalty free, irrevocable, worldwide license to publish this article, to represent this article in derivative works, to disseminate and make available the article to the public for the purposes of education and research, and to permit others to do so.

To whom correspondence should be addressed. Tel.: 718-430-2437; Fax: 718-430-8565; E-mail: hdeng@aecom.yu.edu.
MATERIALS AND METHODS

NADP⁺ was obtained from Roche Molecular Biochemicals, and DHF was purchased from Sigma. [6-13C]DHF was prepared from labeled folate (20). R67 Type II DHFR and its H62C mutant were prepared according to previously published procedures and then dialyzed against deionized water and lyophilized for storage (21). The Raman sample of R67 is prepared by addition of 67 mM phosphate buffer at pH 7 or 100 mM acetate buffer at pH 5.3 to the enzyme powder. The concentrations of R67, DHF, and NADP⁺ were determined using the following extinction coefficients: 63,000 M⁻¹cm⁻¹ at 280 nm for the R67 tetramer, 28,000 M⁻¹cm⁻¹ at 282 nm for DHF, and 18,000 M⁻¹cm⁻¹ at 260 nm for NADP⁺. Typical concentrations of the protein samples were 4 mM. The binary complex of R67 with DHF was prepared by addition of concentrated DHF stock solution to R67 until the final ratio reached 4:4 or 4:8 mM. The binary complex of R67⋅NADP⁺ was similarly prepared. The ternary complex R67⋅NADP⁺⋅DHF was prepared to reach a final concentration of 4:4:4 (mM).

A specially fabricated split-cell cuvette (Hellma Cells) was used to hold the sample. About 25 μl of the ternary complex was loaded into one side of the cuvette, whereas the same amount of binary complex was loaded into the other side. The cuvette was mounted onto a cuvette holder for measurement, maintained at 4°C. About 120 milliwatts of the 568.2 nm line from a Coherent 2000-CR krypton ion laser (Coherent Radiation Inc., Palo Alto, CA) was used to excite Raman scattering. Data were collected by a Mac computer (Apple, Cupertino, CA) interfaced with a CCD detector (Princeton Instruments model LN/CCD-1152UV with a ST-135 CCD controller), which is coupled to a Triplemat spectrometer (Spex Industries, Metuchen, NJ). The spectrum in one side of the split cuvette is taken, the split cell is translated, and the spectrum in the other side is taken. This sequence is repeated until sufficient signal to noise is obtained. A difference spectrum is generated by numerically subtracting the sum of the spectra obtained from each side. In general, the two summed spectra do not subtract to zero, as judged by the subtraction of well known protein marker bands (for example, the amide I, amide III, and the 1450 cm⁻¹ bands, the latter band being especially useful because it is generally not affected by protein conformational changes). These protein marker bands are determined from their bandwidths (generally much broader than those from spectra of bound substrates) and their characteristic positions. Hence, one summed spectrum is scaled by a small numerical factor, generally between 1.05 and 0.95, which is adjusted until the protein bands are nulled. These procedures have been discussed and illustrated many times previously (19).

RESULTS

Fig. 1a shows the Raman spectra of DHF in solution at pH 7. The N5 of the dihydropterin ring is unprotonated at this pH because its pKa is 2.6 (12). It has been shown that the Raman band at 1655 cm⁻¹ is due to the N5=C6 stretch mode of dihydropterin based on its shift upon ¹³C labeling at C6 (10). This band does not shift when DHF is suspended in D₂O as shown in Fig. 1b. When N5 is protonated, the N5=C6 stretch mode of a dihydropterin ring has a higher frequency (~1675 cm⁻¹) and shifts down to 1660 cm⁻¹ upon deuteration of N5 (10). Thus, this mode has been used to identify the protonation states of N5 in dihydropterin-enzyme complexes (10, 11).
assigned to the C6–N5 stretch and the combinations of C2–N1 and C=C stretches of the dihydropterin ring, respectively. The two double bond combination modes of DHF in R67 have nearly the same frequencies and deuterium shifts compared with their corresponding modes in solution (Fig. 2b). The C6–N5 shifts up by 7 cm⁻¹, a small but significant change. It is interesting to point out that this shift is in the opposite direction observed for the \(E.\ coli\) DHFR-DHF-NADP⁺ complex, in which the C6–N5 stretch frequency shifts down by 5 cm⁻¹ (10). We can conclude that N5 of DHF in the R67 ternary complex is unprotonated at pH 7, because this band does not shift upon deuteration of the sample (Fig. 2b).

Previous studies on the binding of various cofactors/substrates using isothermal titration calorimetry and fluorescence have shown that the binding stoichiometry per R67 tetramer is 2. Each active site of tetrameric R67 DHFR can accommodate either two DHF molecules or one DHF and one NADP⁺. There are two \(K_a\) values, 250 and 4.4 \(\mu\)M, respectively, associated with DHF binding to R67, and this binding process displays positive cooperativity. Binding of DHF to the R67/NADP⁺ complex also is positively cooperative with a \(K_a\) of 4.8 \(\mu\)M (22). X-ray crystallographic studies of R67/folate suggest the environments of the two pterin rings from two folate molecules in the binding site are quite different. For example, the N5 of one pterin ring interacts directly with a structural water molecule but there is no such interaction on the other pterin ring (2). Therefore, we conducted additional Raman measurements of several R67:2DHF complexes to determine whether the water molecule that is close to N5 of the dihydropterin ring could affect its protonation state.

Fig. 2. a, Raman difference spectrum from the ternary complex spectrum of R67 with NADP⁺ and DHF ([DHFR-NADP⁺] :[DHF] = 4:4 mM) minus the spectrum of DHFR-NADP⁺ at 4 °C in 50 mM phosphate buffer, pH 7.0. The difference bands are about 10 cm⁻¹, a small but significant change. It is interesting to point out that this shift is in the opposite direction observed for the \(E.\ coli\) DHFR-DHF-NADP⁺ complex, in which the C6–N5 stretch frequency shifts down by 5 cm⁻¹ (10). We can conclude that N5 of DHF in the R67 ternary complex is unprotonated at pH 7, because this band does not shift upon deuteration of the sample (Fig. 2b).
yet it maintains its tetrameric structure at lower pH values. Its catalytic efficiency is even higher than wild type R67 at pH values below 7 (21). Thus, the H62C mutant was used for the Raman studies of DHF bound in R67-NADP⁺-DHF.

Fig. 3a shows the difference Raman spectrum between H62C-NADP⁺-DHF and H62C-NADP⁻ at pH 7. The concentration ratios of the H62C-NADP⁺-DHF and H62C-NADP⁻ complexes were 4:4:4 and 4:4:4, respectively. The two modes at 1560 and 1607 cm⁻¹ due to C2=N1 and C=C stretch combinations do not change much compared with that in solution or in wild type R67. However, there are major changes in the C6=N5 stretch region. Three bands at 1642, 1657, and 1673 cm⁻¹ are observed, whereas only one band near this frequency is observed in solution or in wild type R67. Specifically, the band at 1657 cm⁻¹ is close to the N5-protonated C6=N5 stretch mode of DHF in E. coli DHFR, suggesting a possibility that the N5-protonated DHF may be present in the H62C-NADP⁺-DHF complex. Fig. 3b shows the difference Raman spectrum between H62C-NADP⁺-DHF and H62C-NADP⁻ at pH 5.3. As can be seen from the spectrum, there is no significant change when the sample pH is lowered from pH 7 to 5.3. If the 1673 cm⁻¹ band in Fig. 3a were the N5 protonated C6=N5 stretch mode, the relative intensity ratio of the 1673 cm⁻¹ band to the 1657 cm⁻¹ (or 1642 cm⁻¹) band would be expected to increase by at least an order of magnitude over the pH range. Furthermore, this band does not shift upon deuteration of the sample (Fig. 3c) whereas an ∼15 cm⁻¹ shift toward lower frequency upon deuteration is expected for an N5 protonated C6=N5 stretch mode (10, 11). Thus, it is concluded that the 1673 cm⁻¹ band is not the protonated C6=N5 stretch mode of DHF. Fig. 3c also shows that the other two modes at 1642 and 1657 cm⁻¹ in Fig. 3a show no shift when the sample is deuterated, indicating that there is no significant amount of protonated N5 in the H62C-NADP⁺-DHF complex at pH 5.3. Further studies using

|                  | ecDHFR⁰ | D27S ecDHFR⁰ | R67 | H62C R67 |
|------------------|---------|--------------|-----|----------|
| $k_{cat}$ (s⁻¹)  | 230     | 0.44         | 1.3 | 1.2      |
| $k_{cat}/K_{m}$  | 460⁰    | 0.0031       | 0.22| 0.043    |
| $K_{m}$ (μM)     | 0.5⁰    | 140          | 5.8 | 29       |

⁰ Ref. 4. ⁰ Ref. 13. ¹ Ref. 21. ² Ref. 21. ³ $K_d$ at pH = 6.0 (4).

¹¹C6-labeled DHF show that both bands at 1657 and 1642 cm⁻¹ in the H62C-NADP⁺-DHF complex in Fig. 3b disappear, but the band at 1673 cm⁻¹ remains at the same place (data not shown). Such results indicate that the bands at 1642 and 1657 cm⁻¹ in Fig. 3, a and b, contain significant C6=N5 stretch whereas the 1673 cm⁻¹ band is due to some other high frequency vibrational mode, possibly the C4=N stretch of dihydropterin.

As shown in Fig. 1, the C6=N5 stretch mode is sensitive to the environment of DHF. Thus, two C6=N5 stretches observed for DHF when bound to H62C-NADP⁺ (Fig. 3) suggest the existence of heterogeneous binding for DHF in H62C. In previous studies, ab initio calculations were conducted on a series of model systems of 7,8-dihydropterin derivatives, including its complex with a carboxyl group in contact with −N2H2 and −N3H, a water molecule hydrogen-bonded to C2=O in various configurations, and different orientations of the R group attached to C6 relative to the C6=N5 bond. The results showed that the interaction of a carboxyl group with the N2 and N3 hydrogens has a relatively large effect on the C6=N5 stretch mode, causing a shift of this mode by about 10 cm⁻¹. The hydrogen bonding to C4=O by a water molecule does not have significant effect on the C6=N5 stretch, causing less than 5 cm⁻¹ shift. On the other hand, the orientation of the R group on C6 has a significant effect on the C6=N5 stretch. A rotation of this group around C6-R can cause a 10 cm⁻¹ shift of the C6=N5 stretch. Thus, a combination of all these factors will determine the frequency shift of the C6=N5 stretch observed when DHF binds to H62C. Hence, the most plausible explanation for the split of the C6=N5 stretch mode in H62C mutant is two binding modes for DHF whereby the arms of the C6-R bond adopt different orientations. The difference of hydrogen-bonding patterns on the DHF dihydropterin ring may also contribute to the C6=N5 frequency difference. The apparent heterogeneous nature of the binding site in H62C may well be caused by the heterogeneous nature of the H62C mutant R67. For example, some of the H62C tetramers may contain two disulfide bonds at the dimer interface whereas others may have only one (21). In addition, although substrate inhibition is not noted in wild type R67 DHFR (because of non-productive binding of two DHF molecules), some substrate inhibition is observed in the H62C mutant, indicating there must be some differences in how DHF is bound.

**DISCUSSION**

It has been shown that the pK₅ of N5 of DHF in the DHFR-NADP⁺-DHF ternary complex of the E. coli chromosomal enzyme (ecDHFR), believed to be a structural mimic of the productive DHFR-NADP⁺-DHF complex, is 6.5, which is about four units higher than its solution value of 2.6. In studies of some mutant ecDHFRs, the pK₅ of N5 is substantially reduced (10), and concomitantly, the hydride transfer rate is substantially reduced (see Table I); however, activity is re-
stored at low values of pH (13). All this prompts the suggestion that the enzymic pathway of converting DHF to THF involves first the protonation of N5 and then, in a subsequent step, hydride transfer from NADPH to C6. Raising the pK_a of N5 of bound DHF to 6.5 in the E. coli DHFR ternary complex yields substantial protonated DHF at physiological pH values. This conjecture is reinforced by the finding that the pK_a associated with the hydride transfer rate for E. coli DHFR is also 6.5 (4). From a mechanistic point of view, that the protein environment raises the pK_a of N5 by four pH units to 6.5 in the productive enzyme-catalyzed reaction can proceed without increasing the pK_a of N5 in DHF. Hence, the amount of protonated complex will affect the rate of hydride transfer.

Our current studies of the R67-NADP⁺-DHF complex show that the N5 pK_a of DHF in this complex is not protonated at pH 5.3. Because our Raman technique can detect relative peak intensity changes of 5%, e.g. the relative intensity of the 1657 and 1673 cm⁻¹ bands in Fig. 3, our results indicate that the pK_a of N5 of DHF in the R67-NADP⁺-DHF complex is less that 4. This value is not much increased, if at all, compared with the solution value. In a previous study on the R67-catalyzed, pH-dependent hydride transfer reaction, various models used to fit the pH-dependent k_cat data lead to pK_a values between 2 and 3 (13), consistent with our Raman results. The low apparent pK_a value of the reaction is also found in the D27S mutant E. coli DHFR-NADP⁺-DHF complex, in which the Asp-27 is replaced by serine (10). Because in R67 DHFR there is no comparable active site residue corresponding to Asp-27 in E. coli DHFR, it is interesting to compare R67 DHFR with the E. coli D27S mutant as seen in Table I. The ecDHFR D27S mutant shows a hydride transfer rate that is reduced by over 2 orders of magnitude at pH 7 compared with wild type values (13). In either the wild type or H62C mutant R67 DHFRs, the hydride transfer rate is quite close to that observed in the D27S mutant of E. coli DHFR. Relatively large deuterium kinetic isotope effects in the R67 H62C mutant (³P = 3.6 21) and in the D27S mutant of E. coli DHFR (³P = 3.5 13) indicate that the hydride transfer step is rate-limiting. Furthermore, these isotope effects are independent of pH above 5. Because the pK_a of N5 for DHF in the Michaelis complex of R67 is less than 4 as suggested by our Raman studies, the activity-pH profile can be used to reveal how closely the preprotonation of N5 is correlated with the enzyme-catalyzed reaction.

Assuming the R67-catalyzed hydride transfer reaction occurs after the protonation of N5 of DHF and follows a simple Michaelis-Menten type of reaction mechanism, the k_cat would increase by 1 order of magnitude with every pH unit decrease in the pH range where pH is much higher than the pK_a of N5. In the D27S mutant of ecDHFR, k_cat increases by 0.67 order of magnitude with every pH unit decrease, whereas in H62C R67 DHFR (13) k_cat increases by 0.75 order of magnitude with every pH unit decrease (21). Such a positive correlation is consistent with the model that N5-unprotonated DHF predominates in the Michaelis complex under physiological conditions for the R67 enzyme and the D27S mutant of ecDHFR and that protonation is likely to occur before the hydride transfer step. If that is the case, R67 DHFR may represent a limit of how fast the enzyme-catalyzed DHF to THF reaction can proceed without increasing the pK_a of N5 in DHF.

REFERENCES

1. Matthews, D. A., Smith, S. L., Baccarani, D. P., Burnhall, J. J., Oatley, S. J., and Kraut, J. (1986) Biochemistry 25, 4194–4204.
2. Narayana, N., Matthews, D. A., Howell, E. E., and Xuong, N.-H. (1995) Nat. Struct. Biol. 2, 1018–1025.
3. Reece, L. J., Nichols, R., Ogden, R. C., and Howell, E. E. (1991) Biochemistry 30, 10895–10904.
4. Fierke, C. A., Johnson, K. A., and Benkovic, S. J. (1987) Biochemistry 26, 4085–4092.
5. Dion-Schults, A., and Howell, E. E. (1997) Protein Eng. 10, 263–272.
6. Kraut, J., and Matthews, D. A. (1987) in Biological Macromolecules and Assemblies: Active Sites of Enzymes (Jurnak, F. A., and McPherson, A., eds) Vol. 3, pp. 1–72, John Wiley & Sons, Inc., New York.
7. Blakley, R. L. (1995) Adv. Enzymol. 70, 23–102.
8. Cannon, W. R., Garrison, B. J., and Benkovic, S. J. (1997) J. Am. Chem. Soc. 119, 2386–2395.
9. Li, D., Levy, L. A., Gabel, S. A., Lebetkin, M. S., DeRose, E. F., Wall, M. J., Howell, E. E., and London, R. E. (2001) Biochemistry 40, 4242–4252.
10. Chen, Y.-Q., Kraut, J., Blakley, R. L., and Callender, R. (1994) Biochemistry 33, 7021–7026.
11. Deng, H., and Callender, R. (1998) J. Am. Chem. Soc. 120, 7730–7737.
12. Maharaj, G., Selinsky, B. S., Appleman, J. R., Perlman, M., London, R. E., and Blakley, R. L. (1990) Biochemistry 29, 4554–4560.
13. Howell, E. E., Villafranca, J. E., Warren, M. S., Oatley, S. J., and Kraut, J. (1986) Science 231, 1123–1128.
14. Morrison, J. F., and Stone, S. R. (1988) Biochemistry 27, 5499–5506.
15. Blakley, R. L., Appleman, J. R., Freisheim, J. H., and Jablonsky, M. J. (1993) Arch. Biochem. Biophys. 306, 501–509.
16. Gready, J. E. (1985) Biochemistry 24, 4761–4766.
17. Hennig, M., D’Arcy, A., Hampele, I. C., Page, M. G. P., Oefner, C., and Dale, G. E. (1998) Nat. Struct. Biol. 5, 357–362.
18. Deng, H., Callender, R., and Dale, G. E. (2000) J. Biol. Chem. 275, 30139–30143.
19. Callender, R., and Deng, H. (1994) Annu. Rev. Biophys. Biomol. Struct. 23, 215–245.
20. Selinsky, B. S., Perlman, M. E., London, R. E., Unkefer, C. J., Mitchell, J., and Blakley, R. L. (1990) Biochemistry 29, 1290–1296.
21. Park, H., Zhuang, P., Nichols, R., and Howell, E. E. (1997) J. Biol. Chem. 272, 2252–2258.
22. Bradrick, T. D., Beechem, J. M., and Howell, E. E. (1996) Biochemistry 35, 11414–11424.
23. Nichols, R., Weaver, D., Eisenstein, E., Blakley, R., Appleman, J., Huang, T.-H., Fu-Yung, H., and Howell, E. (1996) Biochemistry 35, 1695–1706.
24. Huennekens, F. M., and Srimgeour, K. G. (1964) in Pteridine Chemistry (Pfliegerer, W., and Taylor, E. C., eds) pp. 355–376, MacMillan, Oxford, United Kingdom.
