Characterization of the Dihydropterin Reductase Activity of Pig Liver Methylenetetrahydrofolate Reductase*

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Pig liver methylenetetrahydrofolate reductase catalyzes the reduction of quinonoid dihydropterins in vitro. Either NADPH or methylenetetrahydrofolate can serve as the electron donor. Methylenetetrahydrofolate reductase can also support phenylalanine hydroxylation in vitro by regeneration of the tetrahydropterin cofactor. These results lend support to the proposal that reduction of methylenetetrahydrofolate proceeds by tautomerization of the 5-iminium cation to form quinonoid 5-methyltetrahydrofolate, which is then reduced to methylenetetrahydrofolate. The Km values for the NADPH-linked reduction of quinonoid dihydropterins are 40, 30, and 20 µM, respectively, while the Km for (6R,S)methylenetetrahydrofolate is 20 µM at pH 7.2 in phosphate buffer. The reduction of quinonoid dihydropterins is inhibited by adenosylmethionine and dihydropteroylhexaglutamate, which are known to modulate methylenetetrahydrofolate reductase activity.

Scheme 1

MATERIALS AND METHODS

CH3-H4folate reductase was purified from pig liver as previously described (3) except that the Affigel-Blue column was washed with 1 mM NADH (2 ml/column bed volume) prior to elution of the reductase with 2 M NaCl. For certain preparations, the enzyme was concentrated by ultrafiltration and chromatographed on Sephacryl S-200 equilibrated with 0.025 M NaCl. For certain preparations, the enzyme was concentrated by ultrafiltration and chromatographed on Sephacryl S-200 equilibrated with 0.025 M NaCl.

Hbioprotein was prepared by catalytic hydrogenation of bioprotein (4) which was obtained from Regis Chemicals; DMPH2, was a gift from Professor Stephen Benkovic or was obtained from Sigma; and H3PGlu6 was prepared as previously described (5). In experiments where [14C]phenylalanine was converted to [14C]tyrosine, the amino acids were separated by chromatography on sheets of Eastman pre-coated cellulose (No. 6064) as described by Kaufman (6). Details of assay procedures are included in the table legends.

Electrophoresis was performed according to Davis (7) and Ornstein (8) using a Hoefer slab gel electrophoresis apparatus. Protein bands were stained with Coomassie blue and activity was localized by formazan deposition in a solution containing 100 µM NADPH, 100 µM quinonoid DMPH2 (prepared immediately before use by oxidation with bromine), 1.2 mM MTT, 0.3 mM EDTA, and 2 µM FAD in 0.05 M Tris/Cl buffer, pH 7.2 (9, 10).

An apparent molecular weight based on the Stokes radius was obtained by chromatography of CH2-H4folate reductase on a Sephacryl S-200 column which had been calibrated with ribonuclease, chymotrypsinogen A, ovalbumin, bovine serum albumin, pig heart lipoprotein dehydrogenase, and human γ-globulin.
RESULTS

CH$_2$-H$_4$folate reductase, purified by chromatography on Sephacryl S-200, had a specific activity range of 1.2 to 1.8 pmol of CH$_2$-H$_4$folate oxidized min$^{-1}$ mg$^{-1}$ at 37°C. These enzyme preparations were purified 4000- to 6000-fold from liver band was stained with either NADPH/MTT or NADPH/MTT/DMPH$_2$. The protein band associated with activity comprised about 20% of the total protein on the gels. The purified enzyme had an $M_r$ of 180,000.

Enzyme which is purified by chromatography on Affigel-Blue, but not chromatographed on Sephacryl S-200, has a specific activity range of 0.4 to 0.6 pmol of CH$_2$-H$_4$folate oxidized min$^{-1}$ mg$^{-1}$.

The data in Table I document the acceptor specificity for the enzyme-catalyzed oxidation of CH$_2$-H$_4$folate. Quinonoid DMPH$_2$ can replace menadione as an electron acceptor. Stor- age of quinonoid DMPH$_2$, leads to spontaneous rearrangement to form the 7,8-dihydro tautomer (12), with a half-life of 39 min under the conditions of these experiments. The decrease in the activity of DMPH$_2$ as an electron acceptor after 60 min suggests that only the quinonoid tautomer is a substrate for reduction. Air oxidation is also known to convert H$_2$pterins to quinoid H$_2$pterins (12). In the presence of air, but not under nitrogen, both DMPH$_2$ and H$_2$folate form products which serve as electron acceptors. Under the conditions of these experiments, the half-life of DMPH$_2$ in air-saturated buffer is about 15 min.

We have also measured the pyridine nucleotide-dependent reduction of quinoid H$_2$pterins, and our data are summarized in Table II. We have compared the absorbance changes seen during enzymatic reduction of DMPH$_2$ generated by two different methods: by using H$_2$O$_2$/peroxidase or by oxidizing DMPH$_2$ with stoichiometric aliquots of bromine (11) immediately prior to the assay. In the latter case, the extinction coefficient which should be used to convert measured absorbance changes at 340 nm to a velocity in moles of substrate reduced min$^{-1}$ in 13,100 M$^{-1}$ cm$^{-1}$, which is the sum of the $e_{340}$ due to NADPH oxidation and the $e_{340}$ due to reduction of quinoid dihydropterin (6900 M$^{-1}$ cm$^{-1}$ (4)). When these measurements were performed at DMPH$_2$ concentrations from 20 to 150 μM, the same absorbance changes were observed with both methods of forming DMPH$_2$, indicating that we should employ an $e_{340}$ of 13,100 M$^{-1}$ cm$^{-1}$ in both cases. The rate of peroxidase-catalyzed oxidation of H$_2$pterins is in the order DMPH$_2$ > H$_2$biopterin > H$_2$folate, and is greatly inhibited by 100 μM NADPH or NADH, the reduced pyridine nucleotide concentration routinely used in dihydropteridine reductase assays (13, 14).

The data in Table II demonstrate the rather marked effect of phosphate buffer on $V_{max}$ and on the $K_s$ for NADPH associated with CH$_2$-H$_4$folate reduction. These rather unusual properties of the reductase are also reflected in the reduction of quinoid DMPH$_2$. These data also indicate that the $K_s$ values for quinoid dihydropterin substrates are comparable in magnitude to the $K_s$ for CH$_2$-H$_4$folate since CH$_2$-H$_4$folate reductase is inhibited by H$_2$folate and its polyglutamate analogues, with the lowest $K_s$ being that for H$_2$PteGlu (4). We have examined the effect of this inhibitor on the reduction of quinoid DMPH$_2$. H$_2$PteGlu is competitive with respect to DMPH$_2$, with a $K_s$ of 45 nm in 50 μM phosphate buffer, pH 7.2. Reduction of 150 μM DMPH$_2$ is almost completely inhibited by 2 μM H$_2$PteGlu. Reduction of quinoid DMPH$_2$ is also inhibited by adenosylmethionine, which is an allosteric inhibitor of CH$_2$-H$_4$folate reductase (2).

### Table I

| Electron acceptor | CH$_2$-H$_4$folate oxidized in 10 min |
|-------------------|------------------------------------|
| Menadione         | 100                                 |
| Quinonoid DMPH$_2$ (160 μM) | 45                                 |
| Quinonoid DMPH$_2$ (enzyme omitted) | 100                                 |
| DMPH$_2$ (184 μM), aerobic | 48                                 |
| DMPH$_2$ (194 μM), under nitrogen | 13                                 |
| H$_2$folate (133 μM), aerobic | 46                                 |
| Oxygen (air-saturated buffer) | 0                                  |

*All assays were performed at 25°C in 50 mM Tris/Cl buffer, pH 7.2, 0.3 mM in EDTA and 2 μM in FAD. Quinonoid DMPH$_2$ was generated immediately before use by oxidation with a stoichiometric quantity of bromine (11). Each assay contained 250 μmol of (1-C-methyl)[H$_4$folate (2000 dpm nmol$^{-1}$) in 0.5 ml. CH$_2$-H$_4$folate oxidation was measured by addition of dimerone at the end of the reaction, heating at 100°C for 2 min, extraction of the dimerone-formaldehyde complex into toluene, and counting in Econofluor scintillation fluid. An aliquot of enzyme contained 0.12 mg of protein (specific activity, 0.4 μmol min$^{-1}$ mg$^{-1}$ at 37°C) was added to each assay.*

### Table II

| Pyridine nucleotide | Buffer* | Electron acceptor | Relative $V_{max}$ | Relative velocity* | $K_s$ electron acceptor | $K_s$ pyridine nucleotide |
|---------------------|---------|-------------------|-------------------|-------------------|-------------------------|-------------------------|
| NADPH               | Phosphate | (6R,S)-CH$_2$-H$_4$folate | 1.0 | 0.86 | 20 | 16 |
| NADH                | Phosphate | (6R,S)-CH$_2$-H$_4$folate | 1.0 | 0.50 | 26 | 100 |
| NADPH               | Tris/Cl | (6R,S)-CH$_2$-H$_4$folate | 0.5 | 0.42 | 40 | 20 |
| NADH                | Tris/Cl | (6R,S)-CH$_2$-H$_4$folate | 0.5 | 0.36 | 770 | 120 |
| NADPH               | Phosphate | q-DMPH$_2$ | 1.1 | 0.98 | 40 | 12 |
| NADH                | Phosphate | q-DMPH$_2$ | 0.5 | 0.5 |
| NADPH               | Phosphate | q-DMPH$_2$ | 0.6 | 0.6 |
| NADPH               | Tris/Cl | q-DMPH$_2$ | 0.5 | 0.5 |
| NADPH               | Tris/Cl | q-DMPH$_2$ | 0.5 | 0.6 |
| NADPH               | Phosphate | q-H$_2$biopter | 0.5 | 0.5 | 30 | 10 |
| NADPH               | Phosphate | q-H$_2$biopter | 0.5 | 0.5 | 20 | 10 |

*Phosphate and Tris buffers were each 0.05 M, pH 7.2, 2 μM in FAD and 0.3 mM in EDTA. Assays were conducted in 1 ml of the indicated buffer. Where quinonoid substrates were used, the assays contained 1 μL of 30% H$_2$O$_2$ and 32 μg of horseradish peroxidase. The order of addition was H$_2$O$_2$, peroxidase, reduced pyridine nucleotide, and H$_2$pterin. The pterin was oxidized to about 60 s and then the nonenzymatic blank rate was measured for 1 to 2 min prior to addition of the enzyme. All values have been corrected for the nonenzymatic blank rate. All lots of enzyme containing 4 μg of protein (specific activity, 1.2 μmol of CH$_2$-H$_4$folate oxidized min$^{-1}$ mg$^{-1}$ at 37°C) were added to each assay.*

*Values have been extrapolated to saturating acceptor in the presence of 100 μM reduced pyridine nucleotide.
Table III compares the stimulation of phenylalanine-dependent NADPH oxidation by CH$_2$-H$_4$folate reductase and dihydropteridine reductase. CH$_2$-H$_4$folate reductase can substitute for dihydropteridine reductase in supporting phenylalanine-dependent NADPH oxidation, and its activity, but not that of sheep liver dihydropteridine reductase, is inhibited by adenosylmethionine. The specificity of the coupled assay for pyridine nucleotide has also been examined. In the presence of CH$_2$-H$_4$folate reductase, 150 µM NADPH and NADH are about equally effective in phosphate buffer, whereas in the presence of sheep liver dihydropteridine reductase, the activity is markedly higher with NADH than with NADPH. 

The data in Table IV show the ability of CH$_2$-H$_4$folate reductase to support phenylalanine hydroxylation, as measured by tyrosine formation. Similar results were obtained when the hydroxylation reaction was followed by measurement of the conversion of labeled phenylalanine to tyrosine. The product of the hydroxylation reaction in the presence of CH$_2$-H$_4$folate reductase and either NADPH or CH$_2$-H$_4$folate was shown to be tyrosine by its co-chromatography with authentic $[^3]$H]tyrosine.

**DISCUSSION**

Since the preparations of CH$_2$-H$_4$folate reductase used for these experiments were not homogeneous, it was important to establish that the quinonoid dihydropterin reductase activity was not due to contaminating NADH-dependent dihydropteridine reductase (EC 1.6.99.10). We have modified our purification procedure to attempt to remove any possible contaminating dihydropteridine reductase. Since dihydropteridine reductase is adsorbed onto columns containing immobilized Cibacron Blue F3GA (the ligand on Affigel-Blue columns) but can be eluted with 10 µM NADH (16), we rinsed our Affigel-Blue columns with 1 mM NADH prior to elution of CH$_2$-H$_4$folate reductase. No NADH or NADPH-dependent dihydropteridine reductase activity has been detected in the NADH eluate. The $M_r$ of dihydropteridine reductase is 42,000 to 50,000 (14, 17), whereas that of CH$_2$-H$_4$folate reductase is about 180,000. Thus, chromatography on Sephacryl S-200 should have separated these two enzymes if both were present. Only one peak of activity is seen on chromatography, corresponding to an $M_r$ of about 180,000. Slab gel electrophoresis shows only one band staining for activity, and no bands are seen on staining with NADH/MTT/DMPH in Tris buffer, conditions which should be optimal for staining of dihydropteridine reductase. Thus, we feel that dihydropteridine reductase is not a contaminant of our preparations. The complete inhibition of NADPH-dependent dihydropterin reductase activity by 2 µM H$_2$PteGlu$_6$, using an enzyme preparation which had not been chromatographed on Sephacryl S-200, also suggests that all the dihydropteridine reductase activity is due to CH$_2$-H$_4$folate reductase.

Recently, Nakanishi and co-workers (18) have described the isolation of a homogeneous NADH-dependent dihydropteridine reductase from bovine liver. This enzyme shows almost complete specificity for NADPH in Tris buffer, as does CH$_2$-H$_4$folate reductase. However, its $M_r$ is about 70,000 and its $K_m$ for NADPH is less than 1 µM, suggesting that this too is a distinct enzyme showing dihydropteridine reductase activity.

One of our interests in testing CH$_2$-H$_4$folate reductase for its ability to support phenylalanine hydroxylation in vitro was to ascertain whether this enzyme might be capable of playing a role in support of H$_2$biopterin-dependent hydroxylation reactions in vivo. The two major tissues where such a role deserves consideration are liver and brain. Liver is the organ where the bulk of phenylalanine hydroxylation occurs. Although there is a large excess of dihydropteridine reductase activity (14) over CH$_2$-H$_4$folate reductase activity in rat liver, the activity of CH$_2$-H$_4$folate reductase appears to exceed that of phenylalanine hydroxylase (19). Lack of dihydropteridine reductase leads to hyperphenylalaninemia as well as to neurological deterioration (20-22). It is not certain to what extent, if any, CH$_2$-H$_4$folate reductase is able to support phenylalanine hydroxylation in these patients. The finding that their hyperphenylalaninemia is not invariably severe (23, 24) and that some of them can tolerate larger amounts of dietary phenylalanine than can patients with classic phenylketonuria (23) strongly suggests that some phenylalanine hydroxylation occurs and hence, that some reduction of quinonoid

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**TABLE III**

| Conditions | N-Adenylmethionine | Phenylalanine oxidation rate $\times$ NADPH oxidation $\times$ Inhibition |
|------------|-------------------|---------------------------|
| CH$_2$-H$_4$folate reductase$^a$ | None | 1.41 | 0 |
| CH$_2$-H$_4$folate reductase$^b$ | 100 | 0.40 | 72 |
| Dihydropteridine reductase$^c$ | None | 2.82 | 0 |
| Dihydropteridine reductase$^d$ | 100 | 2.66 | 6 |

**TABLE IV**

| Experiment | Additions | Tyrosine formed $\mu$mol/20 min |
|------------|-----------|-------------------------------|
| 1          | None      | 20.0                          |
| 2          | NADPH, no reductase | 37.3                         |
| 3          | NADPH, dihydropteridine reductase | 89.1 52.0                   |
| 4          | NADPH, CH$_2$-H$_4$folate reductase | 63.0 25.7                   |
| 5          | CH$_2$-H$_4$folate, no reductase | 19.8                         |
| 6          | CH$_2$-H$_4$folate, CH$_2$-H$_4$folate reductase | 26.6 6.8                   |

$^a$ Tyrosine formation was stimulated by NADPH in the absence of added reductase (compare Experiments 1 and 2). This stimulation was due to the nonenzymatic reduction of quinonoid dihydropteroins by reduced pyridine nucleotides (16). The "reductase-dependent" formation of tyrosine is that amount formed in the presence of both NADPH and reductase less the amount formed in the presence of NADPH but in the absence of reductase.

$^b$ C. Daubner and R. G. Matthews, unpublished data.
H₂biopterin must be going on. A portion of this residual phenylalanine hydroxylation may be supported by CH₂-
H₄folate reductase.

CH₂-H₄folate reductase may also be able to support limited rates of tyrosine and tryptophan hydroxylation in brain, particularly in the absence of dihydropteridine reductase. Such a role could account for the finding that a patient with no immunologically or enzymatically detectable dihydropteridine reductase in brain, who had low rates of turnover of dopamine and serotonin in his central nervous system, nevertheless had detectable amounts of both dopamine and serotonin in a biopsy sample of brain (25).

It will also be interesting to determine whether CH₂-
H₄folate reductase plays any role as a dihydropterin reductase in the biosynthesis of dopamine, norepinephrine, and serotonin under normal conditions. Two patients with homocysteinuria due to deficiency of CH₂-H₄folate reductase have been reported to have lower than normal levels of the metabolites of these neurotransmitters in their cerebrospinal fluids (26). Such findings would be consistent with a role for CH₂-
H₄folate reductase in the synthesis of these neurotransmitters.

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REFERENCES
1. Donaldson, K. O., and Keresztesy, J. C. (1962) J. Biol. Chem. 237, 3815–3819
2. Kutzbach, C., and Stokstad, E. L. R. (1971) Biochim. Biophys. Acta 250, 459–477
3. Matthews, R. G., and Haywood, B. J. (1979) Biochemistry 18, 4845–4851
4. Matthews, R. G., and Baugh, C. M. (1980) Biochemistry 19, 2040–2045
5. Kaufman, S. (1967) J. Biol. Chem. 242, 3934–3943
6. Kaufman, S. (1969) Arch. Biochem. Biophys. 134, 249–252
7. Davis, B. J., Jr. (1964) Ann. N. Y. Acad. Sci. 121, 402–427
8. Ornstein, L. (1964) Am. N. Y. Acad. Sci. 121, 321–349
9. Cotton, R. H., and Jennings, I. (1978) Eur. J. Biochem. 83, 319–324
10. Hasegawa, H. (1977) J. Biochem. (Tokyo) 81, 169–177
11. Dietrich, R., and Benkovic, S. J. (1979) J. Am. Chem. Soc. 101, 6144–6145
12. Kaufman, S. (1961) J. Biol. Chem. 236, 804–810
13. Nielsen, K. M., Simonsen, V., and Lind, K. E. (1969) Eur. J. Biochem. 9, 497–502
14. Craine, J. E., Hall, E. S., and Kaufman, S. (1972) J. Biol. Chem. 247, 6082–6081
15. Udenfriend, S., and Cooper, J. R. (1952) J. Biol. Chem. 196, 227–233
16. Scrimgeour, K. G., Tirpak, A., and Chauvin, M. M. (1979) in Chemistry and Biology of Pteridines (Kisliuk, R. L., and Brown, G. M., eds) pp. 207–210, Elsevier/North Holland, New York
17. Cheema, S., Soldin, S. J., Knapp, A., Hofmann, T., and Scrimgeour, K. G. (1973) Can. J. Biochem. 1229–1239
18. Nakanishi, N., Hasegawa, H., and Watabe, S. (1977) J. Biochem. (Tokyo) 81, 681–685
19. Milstien, S., and Kaufman, S. (1975) J. Biol. Chem. 250, 4782–4785
20. Kaufman, S. (1979) in Chemistry and Biology of Pteridines (Kisliuk, R. L., and Brown, G. M., eds) pp. 117–124, Elsevier/North Holland, New York
21. Kaufman, S., Holtzman, N. A., Milstien, S., Butler, I. J., and Krumholz, A. (1975) N. Engl. J. Med. 293, 785–790
22. Milstien, S., and Kaufman, S. (1975) Biochem. Biophys. Res. Commun. 66, 475–481
23. Grobe, H., Bartholome, K., Milstien, S., and Kaufman, S. (1978) Eur. J. Pediatr. 129, 93–98
24. Brewster, T. G., Moskowitz, M. A., Kaufman, S., Breslow, J. L., Milstien, S., and Abroms, I. F. (1979) Pediatrics 63, 94–99
25. Butler, I. J., Koslow, S. H., Krumholz, A., Holtzman, N. A., and Kaufman, S. (1978) Ann. Neurol. 3, 224–230
26. Singer, H. S., Butler, I., Rothenberg, S., Valle, D., and Freeman, J. (1980) Neurology 30, 419