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Biochemistry

α-Pyridine nucleotides as substrates for a plasmid-specified dihydrofolate reductase*

[β-NAD(P)H/pyridine nucleotide analogues/bacterial dihydrofolate reductase/dehydrogenases/trimethoprim resistance]

SHEILA L. SMITH AND JAMES J. BURCHALL

Microbiology Department, Wellcome Research Laboratories, Research Triangle Park, North Carolina 27609

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ABSTRACT **The α epimers of pyridine nucleotides are almost totally inactive as reductants in dehydrogenase reactions. In contrast, the R plasmid R67-specified dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) isolated from trimethoprim-resistant *Escherichia coli* utilized α-NADPH and α-NADH in addition to the "normal" β-epimers. The enzymes from bacterial and mammalian sources used only β-NADPH and β-NADH. The K_m value for α-NADPH (16 μM) was 4-fold greater than that for β-NADPH (4 μM), while the maximal velocity of the α-NADPH-catalyzed reaction was 70% of that seen with the β-NADPH. β-NADP$^+$ and α-NADP$^+$ were competitive inhibitors of the R67 enzyme. Pyridine nucleotide analogues such as deamino- and acetyl-NADPH were used readily by bacterial, plasmid, and mammalian enzymes, whereas thio-NADPH was used only by the plasmid enzyme. These data suggest that the enzyme from R plasmid R67 possesses a pyridine nucleotide binding site different from that of other dihydrofolate reductases and dehydrogenases.**

Strains of *Escherichia coli* that are resistant to high levels of trimethoprim contain a novel, R-plasmid-specific, dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) in addition to the normal chromosomal DHFR (1–3). The R plasmid R67 enzyme has K_m values for dihydrofolate and NADPH that are similar to those of the chromosomal DHFR. However, unlike the chromosomal enzyme, it is insensitive to a variety of inhibitors, including methotrexate, pyrimethamine, and trimethoprim (3). In previous studies, we have shown that the R plasmid enzyme differs markedly from other DHFRs (4, 5) and its amino acid sequence shows almost no homology with the published sequences of other bacterial and mammalian DHFRs (ref. 6; see table 4 of ref. 7). Moreover, the enzyme from plasmid R67 is a tetramer composed of four chemically identical subunits, whereas all known DHFRs from bacterial and mammalian sources are monomers. Binding studies demonstrated that the tetramer contains four folate binding sites but only one NADPH site (8). In this communication, we report another unique property of the plasmid DHFR, its capacity to utilize α-NADPH as a reductant. These and other data obtained from studies with cofactor analogues suggest that this enzyme possesses a pyridine nucleotide binding site not previously noted in dehydrogenases (9).

MATERIALS AND METHODS

Biochemicals and Enzymes. NADPH, NADP$^+$, NADH, and NAD$^+$ epimers, nicotinamide-hypoxanthine dinucleotide phosphate (NHDPH), oxidized 3-acetylpyridine-adenine dinucleotide phosphate (AcPyADP$^+$), oxidized thionicotinamide-adenine dinucleotide phosphate (TNADP$^+$), β-NMNH, α-ketoglutaric acid, glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP$^+$ 1-oxidoreductase, EC 1.1.1.49) from yeast, glucose 6-phosphate, isocitrate dehydrogenase [*threo*-D$_S$-isocitrate:NADP$^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] from pig heart, DL-isocitric acid, lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) from rabbit muscle, pyruvic acid, and ATP are all products of Sigma. Glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3) from beef liver was from Miles. Formyltetrahydrofolate synthetase [formate:tetrahydrofolate ligase (ADP-forming), EC 6.3.4.3] from *Clostridium acidi-urici* was kindly supplied by Jesse Rabinowitz (Univ. of California, Berkeley). DHFR from *Lactobacillus casei* was obtained from the Enzyme Center (Boston). R67 DHFR was purified as described (4). Other DHFRs were kindly supplied from within the Wellcome Research Laboratories: purified from L1210 murine lymphoma, J. Raper and J. Dann; purified from *E. coli* RT500 forms 1 and 2, D. Baccanari; purified from rat liver, *Proteus vulgaris*, and *Crithidia fasciculata*, R. Ferone; and partially purified from *Neisseria gonorrhoeae*, R. Tansik. Dihydrofolate was prepared as described by Blakley (10) and stored in 5 mM HCl and 50 mM 2-mercaptoethanol at −80°C.

DHFR Assay. DHFR was assayed in 50 mM potassium phosphate, pH 7, at 30°C. Standard assay mixtures contained 70 μM NADPH, 45 μM dihydrofolate, 12 mM 2-mercaptoethanol, and sufficient enzymic protein to cause an A_{340} change of 0.01–0.10 per 5 min. For kinetic determinations dihydrofolate was 22 μM, 2-mercaptoethanol was 6 mM, and the pyridine nucleotide substrate was 0.5 K_m to 80 μM when the K_m was less than 10 μM or 0.2 K_m to 500 μM when the K_m was higher. When α-NADH, β-NADH, or β-NMNH was used as substrate, 0.5-cm black-masked cuvettes were used to reduce A_{340} by half. One enzyme unit is the quantity of enzyme required to convert 1 μmol of NADPH and dihydrofolate to NADP$^+$ and tetrahydrofolate per min calculated based on an absorption coefficient of 12,300 liter mol^{-1} cm^{-1} at 340 nm (11). Preparations of α-NADPH that contained residual β-NADPH were treated by addition of pyruvate (0.5 mM) and lactate dehydrogenase (4.5 units/ml) to oxidize β-NADPH to β-NADP$^+$ before dihydrofolate and DHFR were added. In other experiments glutamate dehydrogenase (2 units/ml) was substituted for lactate dehydrogenase, and NH_4Cl (0.2 mM) and α-ketoglutarate (0.2 mM) were used with the ap-

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Abbreviations: DHFR, dihydrofolate reductase; NMNH, nicotinamide mononucleotide, reduced; NHDPH, nicotinamide-hypoxanthine dinucleotide phosphate, reduced; TNADP$^+$, thionicotinamide-adenine dinucleotide phosphate, oxidized; TNADPH, thionicotinamide-adenine dinucleotide phosphate, reduced; AcPyADP$^+$, 3-acetylpyridine-adenine dinucleotide phosphate, oxidized; AcPyADPH, 3-acetylpyridine-adenine dinucleotide phosphate, reduced.

* Preliminary results of this study were presented at the meeting of the Federation of American Societies for Experimental Biology held May 31–June 4, 1981, in St. Louis, MO (41).

propriate quantity of α-NADPH. When a comparison of the epimers was made, pyruvate or NH_4Cl and α-ketoglutarate were added to the reaction mixture with β-NADPH to equalize salt effects upon the enzymic reaction. *E. coli* DHFR (1 unit/ml) was also used to oxidize β-NADPH to β-$NADP^+$. The method of Horecker and Kornberg (12) was used to determine the absorbance change of the DHFR reaction mixture with α- or β-NADPH to ascertain whether there was a difference between the epimers (13, 14). Dihydrofolate was the limiting substrate at 10 or 25 μM, α- or β-NADPH was 70 μM, and R67 DHFR was 2.5 μM. The concentration of dihydrofolate had been previously determined by its A_{283} and by depletion with *E. coli* DHFR (15). NADPH and NADH concentrations were calculated by using an absorption coefficient of 6,220 liter mol^{-1} cm^{-1} at 340 nm (12). Kinetic data were analyzed by the method of Cleland (16).

Determination of Enzymically Active Products. Coupled reactions with glucose-6-phosphate dehydrogenase were used to demonstrate the presence of the putative product, β-$NADP^+$. Dihydrofolate was 35 or 70 μM, R67 DHFR was 0.08 unit (0.08 mg), and NADPH was 35 or 70 μM. After the DHFR reaction was completed, the total decrease in A_{340} was recorded for a 1-ml reaction mixture, then glucose 6-phosphate (10 mM) and glucose-6-phosphate dehydrogenase (5 units) were added. The resulting increase in A_{340} was recorded.

When the DHFR assay was coupled to the formyltetrahydrofolate synthetase assay to determine enzymically active tetrahydrofolate, the reaction was allowed to deplete dihydrofolate (36 or 72 μM), then the mixture was made 1 M with respect to 2-mercaptoethanol. Samples (100–500 μl) containing 8–40 nmol of tetrahydrofolate were immediately added to screw-capped test tubes containing triethanolamine at pH 8 (100 mM), $MgCl_2$ (10 mM), sodium formate (40 mM), ATP at pH 8 (5 mM), and sufficient H_2O for a final volume of 1 ml. The reaction mixture was incubated 2 min at 37°C, then formyltetrahydrofolate synthetase (2 units) was added. Incubation continued for 10 min at 37°C before the reaction was stopped by addition of 2 ml of 0.36 M HCl. After 30 min at room temperature the A_{350} was recorded and the tetrahydrofolate that had been converted to 5,10-methenyltetrahydrofolate was calculated by using an absorption coefficient of 24,900 liter mol^{-1} cm^{-1} at 350 nm (J. Rabinowitz, personal communication).

Preparation of AcPyADPH and TNADPH. AcPyADPH and TNADPH were prepared by the procedure described by Spector *et al.* (17). AcPyADP^+ or TNADP^+ (6 μmol), glucose 6-phosphate (10 μmol), glucose-6-phosphate dehydrogenase (3 units), and Tris·HCl, pH 8 (50 μmol), were mixed in a total volume of 1 ml, then incubated at 30°C for 3 min. The increase in A_{363} or A_{400} was recorded, and the resulting NADPH concentration was calculated by using the published molar absorption coefficients (liter mol^{-1} cm^{-1}) of 13,600 at 260 nm and 9,100 at 363 nm for AcPyADPH (18) and 15,000 at 260 nm and 11,300 at 400 nm for TNADPH (18, 19). The mixture was then applied to a DEAE-cellulose column (1 × 15 cm) as described by Dunn *et al.* (20). The products were determined to be essentially pure by comparison of their molar absorption coefficients with the published values, by the presence of a single spot when a sample was chromatographed on Whatman 3 MM cellulose in sodium/potassium phosphate, pH 7 (100 mM), and by depletion of the NADPH analogue by R67 DHFR. The enzymic oxidations were performed by incubating AcPyADPH or TNADPH (15 μmol), dihydrofolate (45 μmol), and R67 DHFR (0.08 unit) in a total volume of 1 ml containing potassium phosphate (10 μmol), pH 7. The concentration of NADPH analogue was calculated from the net absorbance change at 363 nm or 400 nm, using the published molar absorption coefficients for the NADPH. Neither dihydrofolate nor tetrahydrofolate influenced the absorbance at these wavelengths. The purified products could be stored at 4°C for at least 2 weeks without detectable changes in absorbance.

Determination of Transhydrogenase Activity. Electron-transfer experiments for transhydrogenase activity were performed as described by Huennekens *et al.* (21). R67 DHFR (6–12 μmol), NADPH (10 nmol), AcPyADP (30 nmol), dihydrofolate (1–5 nmol), and Tris·HCl, pH 7 (50 μmol) were mixed in a total volume of 1 ml. An absorbance spectrum of this reaction mixture was compared to that of the same reaction mixture without AcPyADP, with special attention directed toward changes at 363 nm.

RESULTS

DHFR Activity With α- and β-NAD(P)H. No reaction was detected when the α epimers were tested as substrates with DHFR from *E. coli*, *L. casei*, *N. gonorrhoeae*, *P. vulgaris*, rat liver, L1210 murine lymphoma, or *C. fasciculata*, even at 25,000- to 50,000-fold greater enzyme concentrations than were used to detect the reaction with the β epimers. However, both α-NADPH and α-NADH served as substrates for the R67 R plasmid DHFR (Table 1). The reaction velocities, as measured by decrease in A_{340}, approached those of the β epimers. When α-NADPH was employed as the substrate for the plasmid DHFR the V_{max} was 70% of the β-NADPH value. α-NADPH had a K_m of 16 μM compared to 4 μM for β-NADPH (Fig. 1). Because α-NADPH preparations often contain some β-NADPH as a contaminant, the α-NADPH solutions were pretreated with lactate dehydrogenase, glutamate dehydrogenase, or *E. coli* DHFR to oxidize the β-NADPH. The K_m and V_{max} values were essentially the same regardless of the method used to remove the β-NADPH. In all cases the β-NADPH represented <7% of the total NADPH.

Enzymic Characterization of R67 DHFR Products. Because of the unique properties of the R67 DHFR observed in these experiments, it was considered prudent to examine the enzymic reaction products to exclude any possibility that we were, in fact, studying a reaction similar but not identical to that catalyzed by the bacterial and mammalian enzymes. The UV spectrum of each of the epimers showed similar absorption profiles except that the peak of absorbance of the α epimer occurred at 346 nm rather than 340 nm as observed with the β-NADPH (Fig. 2). In separate experiments equimolar quantities of α- and β-NADPH were allowed to become totally depleted in the presence of the R67 DHFR and the total decrease in A_{340} was the same in each case. Thus, the absorbance values for each epimer were identical at 340 nm. In further experiments, the products of the reaction were shown to be tetrahydrofolate and either α- or β-$NADP^+$ as appropriate. The stoichiometry of

Table 1. DHFR activity with pyridine nucleotide analogues

	R67		*E. coli* RT-500		L1210	
Substrate	Activity, nmol/min	Protein, μg	Activity, nmol/min	Protein, μg	Activity, nmol/min	Protein, μg
β-NADPH	1.15	1.2	0.41	0.01	1.38	0.12
α-NADPH	0.57	1.2	<0.01	100	<0.01	600
β-NADH	0.40	12	0.28	0.10	0.22	0.12
α-NADH	0.49	12	<0.01	100	<0.01	600

Activities are amount of product formed per min, calculated by using a molar absorbance coefficient of 12,300 liter mol^{-1} cm^{-1}. All values were determined in 50 mM potassium phosphate, pH 7, at 30°C. Dihydrofolate was 22 μM, 2-mercaptoethanol was 6 mM, and NADPH or its analogue was held constant at 70 μM.

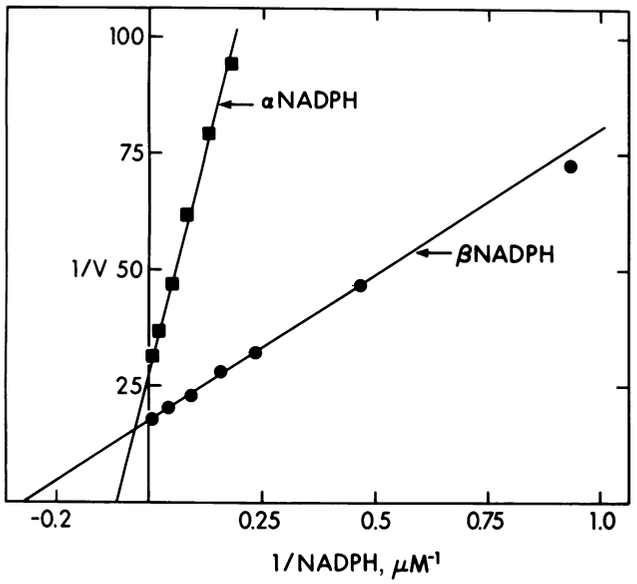

FIG. 1. Comparison of DHFR activity of plasmid R67 when the concentration of α-NADPH (■) or β-NADPH (●) was varied. Enzyme concentration was 26 nM. The v was net A_{340} change per 5 min. Buffer was 10 mM potassium phosphate, pH 7, containing 0.5 mM pyruvate (see *Materials and Methods* for details when α-NADPH was used).

substrate depletion and product appearance as measured in a typical experiment is shown in Table 2. The quantity of α- and β-NADPH depleted in the reaction was calculated from the total decrease in A_{340}. In the case of β-NADP this figure was verified by observing the increase in A_{340} that occurred in the presence of glucose 6-phosphate and glucose-6-phosphate dehydrogenase. In addition, the total quantity of tetrahydrofolate formed was calculated and its presence was verified by converting tetrahydrofolate to 5,10-methenyltetrahyhdrofolate by addition of formyltetrahydrofolate synthetase. The discrepancy in the amount of dihydrofolate consumed and tetrahydrofolate produced in the presence of α-NADPH may indicate that the

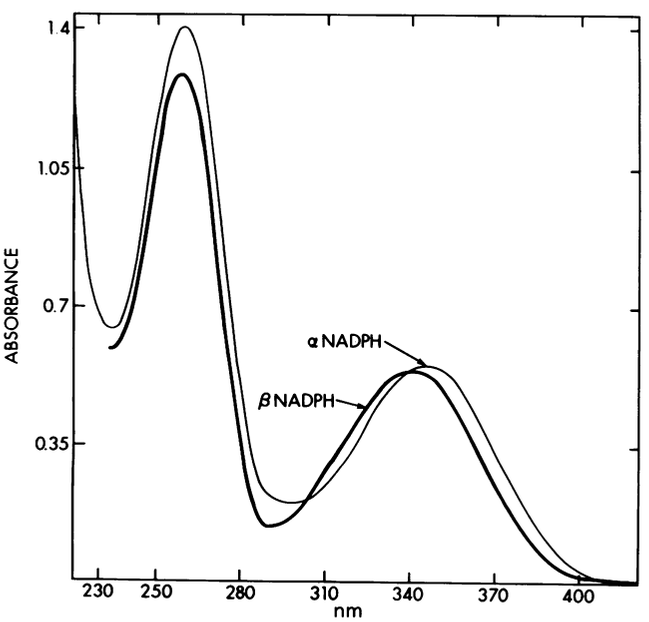

FIG. 2. Comparison of the spectra of β-NADPH and α-NADPH. For each solution 3.2 mg of NADPH was weighed and dissolved in 1 ml of 10 mM potassium phosphate, pH 7. Dilutions were made in the same buffer.

Table 2. Stoichiometry of product formation by R67 DHFR

Substrate	nmol	Product	nmol
α-NADPH	36*	α-NADP	27,† 0‡
Dihydrofolate	35§	Tetrahydrofolate	27,† 33¶
β-NADPH	35*	β-NADP	35,† 35‡
Dihydrofolate	35§	Tetrahydrofolate	35,† 32¶

* Determined by total A_{340}.
† Determined by decrease in A_{340}.
‡ Determined by activity with glucose-6-phosphate dehydrogenase.
§ Determined by enzymic depletion with *E. coli* DHFR.
¶ Determined by activity with formyltetrahydrofolate synthetase.

reaction was pulled to completion only in the presence of formyltetrahydrofolate synthetase. There was no decrease in A_{340} when components of either the glucose-6-phosphate dehydrogenase or isocitrate dehydrogenase systems were added to the products of the DHFR reaction with α-NADPH.

Inhibition of R67 DHFR by α- and β-NADP⁺. Although DHFR cannot catalyze the oxidation of tetrahydrofolate by NADP, the binding of $NADP^+$ could be studied by its inhibition of the forward reaction. When either α- or β-$NADP^+$ was used as an inhibitor of the R67 DHFR, the resulting $1/v$ vs. 1/NADPH plot was linear. Each inhibitor was competitive with NADPH and the K_i value for β-$NADP^+$ (7 μM; data not shown) was 1/3rd of that for α-$NADP^+$ (21 μM) (Fig. 3).

Comparison of the Activity of Pyridine Nucleotide Analogues. The specificity of the pyridine nucleotide binding site was investigated in further experiments using other pyridine nucleotide analogues (Table 3). A study was made to determine the ability of the R67 enzyme to use pyridine nucleotide analogues without the 2′-phosphate group (NADH) or adenosine 2′,5′-phosphate (NMNH) as well as those with substitutions on the nicotinamide (AcPyADPH and TNADPH) and adenine (NHDPH) moieties. The V_{max} values for α-NADH, β-NMNH, and TNADPH of the R67 DHFR were 14–25% of the V_{max} values for β- or α-NADPH, whereas the V_{max} values for β-NADH and AcPyADPH were 2–9% of those for the NADPH epimers. The L1210 enzyme required the β-nicotinamide-ribose moiety for detectable catalytic activity, but the difference between the V_{max} values with NHDPH and β-NMNH was only 5-fold. In-

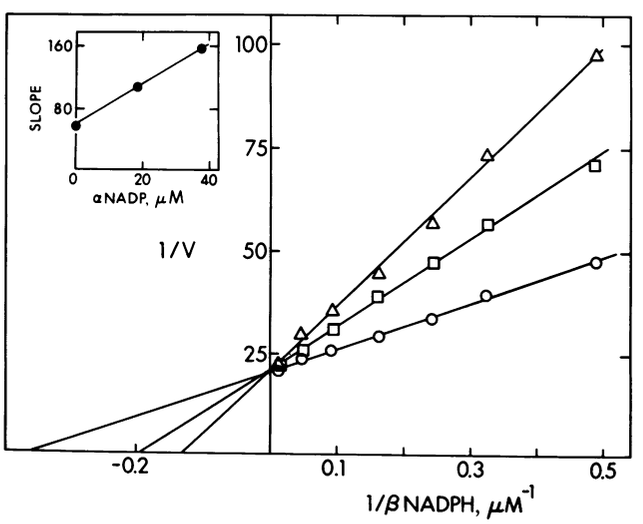

FIG. 3. β-NADPH vs. α-$NADP^+$ with plasmid R67 DHFR. Enzyme concentration and the units of velocity (v) were the same as in Fig. 1. Buffer was 10 mM potassium phosphate, pH 7. α-$NADP^+$ was 0 μM (○), 19 μM (□), and 38 μM (△). (*Inset*) Plot of α-NADP concentration vs. the slope [in units of $(\Delta A_{340}/5\ \text{min})^{-1}$ μM].

Table 3. Kinetic constants for DHFRs

Substrate	R67		*E. coli* RT-500		L1210	
	K_m, μM	V_{max}, relative	K_m, μM	V_{max}, relative	K_m, μM	V_{max}, relative
β-NADPH	3.9	100	2.7	100	5.3	100
α-NADPH	16.6	68	—*	—	—*	—
β-NADH	82	6	235	20	298	100
α-NADH	490	17	—*	—	—*	—
β-NMNH	392	17	1,222	0.1	493	40
NHDPH	6.5	80	3.6	50	5.8	210
AcPyADPH	10.3	2	1.3	8	6.9	100
TNADPH	9.4	14	—*	—	—*	—

All values were determined in 50 mM potassium phosphate, pH 7, at 30°C. Dihydrofolate was 22 μM and concentrations of NADPH analogue varied. Values represent an average of six determinations for β-NADPH and α-NADPH. All other values represent an average of at least two determinations. The coefficient of variation was 20–38%.

* No reaction at 5,000- to 10,000-fold more enzymic protein than was used to determine the V_{max} with β-NADPH.

creased catalytic activity of NHDPH by the L1210 DHFR was unexpected, as were the rates with β-NADH and AcPyADPH. The enzyme purified from rat liver showed the same increased rates of catalysis. On the other hand, the *E. coli* DHFR was considerably more specific in its requirement for the adenosine-ribose moiety with a decrease to 1/10,000th in V_{max} values in its absence.

Transhydrogenase Effect on This Study. No transhydrogenase activity could be demonstrated with the R67 enzyme. Although the protein concentration in this experiment was less than that used by Huennekens and co-workers (21) to demonstrate transhydrogenase activity by chicken liver and L1210 DHFR, it was higher than in any of the other experiments in the present study.

DISCUSSION

This report demonstrates that a highly purified enzyme can effectively utilize the α epimers of pyridine nucleotides. Lactate dehydrogenase, both H_4 and M_4 isozymes from chicken liver, and alcohol dehydrogenase from yeast and horse liver can be shown to utilize the α epimer, under certain conditions. However, it is only 1/1,000th to 1/10,000th as effective as the β epimer (22). The α epimer binds weakly to glutamate dehydrogenase but cannot serve as a catalytically effective substrate for the enzyme (23). In this study, no other DHFR of bacterial, protozoan, or mammalian origin utilized α-NADPH or α-NADH as a substrate at any concentration. When the oxidized α epimer was tested for its ability to inhibit the R67 DHFR, it was only slightly less effective than the β epimer. On the other hand, analogues of β-NADPH with substitutions on the nicotinamide or adenosine were less catalytically effective than NADPH as substrates for the R plasmid enzyme. K_m values with β-NADH for the enzymes from *E. coli* and L1210 essentially agree with previously reported values (24–28).

The unusual catalytic properties of the plasmid enzyme prompted a careful examination of the stoichiometry of the products of the reaction. The data obtained in these studies are consistent with those obtained with bacterial and mammalian DHFR in that 1 mol of β-$NADP^+$ is produced per mol of β-NADPH depleted, and 1 mol of tetrahydrofolate is produced per mol of dihydrofolate depleted when β-NADPH is employed as the reductant (29–33). Similarly, about 1 mol of tetrahydrofolate was produced per mol of dihydrofolate consumed when α-NADPH was the substrate. The fact that β-$NADP^+$ produced in the reaction served as a substrate for glucose-6-phosphate dehydrogenase and that the tetrahydrofolate produced in the reaction served as a substrate for formyltetrahydrofolate synthetase argues strongly that the products of these reactions are the conventionally accepted forms. Comparison of similar stoichiometry of α-$NADP^+$ to α-NADPH was not possible because there is no enzyme known to catalyze this reaction (13, 34).

The biological role of α-NADPH is not well understood (22, 35). It has been reported that enzymically generated and chemically synthesized preparations of NAD can contain as much as 20% α epimer (22, 36–38). In addition, both α and β epimers have been reported in cell extracts (36, 39, 40). Because epimerization can occur even at low temperatures, the presence of α-NADPH in cell extracts may be artifactual. Part of the difficulty in resolving this problem arises from the fact that there was no specific enzymic method until now for the identification of the α epimer (34, 39). The R67 DHFR may provide a useful tool for further investigations regarding the biological prevalence and significance of α epimers of pyridine nucleotides.

The origin of the R67 R plasmid DHFR remains obscure. Previous studies of the amino acid sequence of the R67 DHFR rule out the possibility that it is a simple variant of chromosomal DHFR. We have suggested previously that this enzyme may have arisen from an oxidoreductase unrelated to the well-characterized bacterial and mammalian DHFRs (4). Should such a "precursor" enzyme exist these data suggest that its specificity was lost also at the pyridine nucleotide binding site. Alternatively, it may be that a unique pyridine nucleotide binding site has been created by the interaction of the four subunits. The nature of this binding site can be studied by x-ray crystallography.

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1. Amyes, S. G. B. & Smith, J. T. (1974) *Biochem. Biophys. Res. Commun.* **58**, 412–418.
2. Sköld, O. & Widh, A. (1974) *J. Biol. Chem.* **249**, 4324–4325.
3. Pattishall, K. H., Acar, J., Burchall, J. J., Goldstein, F. W. & Harvey, R. J. (1977) *J. Biol. Chem.* **252**, 2319–2323.
4. Smith, S. L., Stone, D., Novak, P., Baccanari, D. P. & Burchall, J. J. (1979) *J. Biol. Chem.* **254**, 6222–6225.
5. Matthews, D. A., Kraut, J., Smith, S. L. & Burchall, J. J. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 1771 (abstr.).
6. Stone, D. & Smith, S. L. (1979) *J. Biol. Chem.* **254**, 10857–10861.

7. Hitchings, G. H. & Smith, S. L. (1980) *Adv. Enzyme Regul.* **18,** 349–371.
8. Smith, S. L. & Burchall, J. J. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39,** 1771 (abstr.).
9. Kaplan, N. O. (1960) in *The Enzymes,* eds. Boyer, P. D., Lardy, H. & Myrbäck, K. (Academic, New York), 2nd Ed., Vol. 3, pp. 105–169.
10. Blakley, R. L. (1960) *Nature (London)* **188,** 231–232.
11. Hillcoat, B. L., Nixon, P. F. & Blakley, R. L. (1967) *Anal. Biochem.* **21,** 178–189.
12. Horecker, B. L. & Kornberg, A. (1948) *J. Biol. Chem.* **175,** 385–390.
13. Suzuki, K., Nakano, H. & Suzuki, S. (1967) *J. Biol. Chem.* **242,** 3319–3325.
14. Pfleiderer, G., Woenckhaus, C. & Nelböck-Hochstetter, M. (1965) *Justus Liebigs Ann. Chem.* **690,** 170–176.
15. Baccanari, D. P. & Joyner, S. S. (1981) *Biochemistry* **20,** 1710–1716.
16. Cleland, W. W. (1963) *Nature (London)* **198,** 463–465.
17. Spector, T., Jones, T. E. & Miller, R. L. (1979) *J. Biol. Chem.* **254,** 2308–2315.
18. Kaplan, N. O., Ciotti, M. M. & Stolzenbach, F. E. (1956) *J. Biol. Chem.* **221,** 833–844.
19. P-L Biochemicals (1971) *Ultraviolet Absorption Spectra of Pyridine Nucleotide Coenzymes and Coenzyme Analogues* (P-L Biochemicals, Milwaukee, WI), pp. 1–13.
20. Dunn, S. M. J., Batchelor, J. G. & King, R. W. (1978) *Biochemistry* **17,** 2356–2364.
21. Huennekens, F. M., Mell, G. P., Harding, N. G. L., Gundersen, L. E. & Freisheim, J. H. (1970) in *Chemistry and Biology of Pteridines,* eds. Iwai, K., Akino, M., Goto, M. & Iwanami, Y. (International Academic Printing, Tokyo, Japan), pp. 329–350.
22. Oppenheimer, N. J. & Kaplan, N. O. (1975) *Arch. Biochem. Biophys.* **166,** 526–535.
23. Koberstein, R., Krause, J. & Sund, H. (1973) *Eur. J. Biochem.* **40,** 543–548.
24. Morales, D. R. & Greenberg, D. M. (1964) *Biochim. Biophys. Acta* **85,** 360–376.
25. Bertino, J. R., Perkins, J. P. & Johns, D. G. (1965) *Biochemistry* **4,** 839–846.
26. Kaufman, B. T. & Gardiner, R. C. (1966) *J. Biol. Chem.* **241,** 1319–1328.
27. Baccanari, D., Phillips, A., Smith, S., Sinski, D. & Burchall, J. (1975) *Biochemistry* **14,** 5267–5273.
28. Barbehenn, E. K. & Kaufman, B. T. (1980) *J. Biol. Chem.* **255,** 1978–1984.
29. Osborn, M. J. & Huennekens, F. M. (1958) *J. Biol. Chem.* **233,** 969–974.
30. Bertino, J. R., Gabrio, B. W. & Huennekens, F. M. (1960) *Biochem. Biophys. Res. Commun.* **3,** 461–465.
31. Pastore, E. J. & Friedkin, M. (1962) *J. Biol. Chem.* **237,** 3802–3810.
32. Blakley, R. L., Ramasastri, B. V. & McDougall, B. M. (1963) *J. Biol. Chem.* **238,** 3075–3079.
33. Zakrzewski, S. F. (1966) *J. Biol. Chem.* **241,** 2957–2961.
34. Bernofsky, C. (1980) *Methods Enzymol.* **66,** 87–90.
35. Jacobson, E. L., Jacobson, M. K. & Bernofsky, C. (1973) *J. Biol. Chem.* **248,** 7891–7897.
36. Kaplan, N. O., Ciotti, M. M., Stolzenbach, F. E. & Bachur, N. R. (1955) *J. Am. Chem. Soc.* **77,** 815–816.
37. Haynes, L. J., Hughes, N. A., Kenner, G. W. & Todd, A. (1957) *J. Chem. Soc.* 3727–3732.
38. Hughes, N. A., Kenner, G. W. & Todd, A. (1957) *J. Chem. Soc.* 3733–3738.
39. Suzuki, S., Suzuki, K., Imai, T., Suzuki, N. & Okuda, S. (1965) *J. Biol. Chem.* **240,** 554–556.
40. Ricci, C., Pallini, V. & Martelli, P. (1965) *Biochem. Biophys. Res. Commun.* **19,** 296–299.
41. Smith, S. L. & Burchall, J. J. (1981) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **40,** 1688 (abstr.).