INTERCONVERSION OF THE MULTIPLE FORMS OF DIHYDROFOLATE REDUCTASE FROM AMETHOPTERIN-RESISTANT *LACTOBACILLUS CASEI**

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SUMMARY: Dihydrofolate reductase from amethopterin-resistant *Lactobacillus casei* can be separated into two principal forms (I and II) by electrophoresis on polyacrylamide or by chromatography on ion-exchangers. Form (II)(the component that migrates more rapidly toward the positive electrode during electrophoresis at pH 8.5) contains approximately 1 mole of TPNH per mole of protein (MW = 15,000), as judged by spectral characteristics (absorbance maxima at 274 and 340 mμ and fluorescence maximum at 445 mμ) of the intact protein and by analysis (thin-layer chromatography and coenzymatic activity with yeast glutathione reductase) of the nucleotide released by heat denaturation of the protein. Treatment of (II) with 7,8-dihydrofolate results in oxidation of the bound TPNH, release of the resultant TPN, and creation of form (I). (II) can be resynthesized by incubation of (I) with TPNH at neutral pH.

Dihydrofolate reductases from several sources have been resolved by electrophoresis or by ion-exchange chromatography into multiple forms (1,2), designated as (I), (II), etc., with respect to their increasing mobility toward the positive electrode during electrophoresis at pH 8.5. Each form catalyzes the same overall reaction (equation 1) and no differences have yet been found

$$7,8\text{-Dihydrofolate} + \text{TPNH} + H^+ \rightarrow \text{Tetrahydrofolate} + \text{TPN}^+ \qquad (1)$$

in their kinetic parameters. Physiological control of these forms is suggested by their dissimilar increase in successive generations of L1210 cells that are being exposed continuously to amethopterin (3). Some indication of the *structural* basis for this polymorphism has been obtained from previous studies

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(2,4) with the chicken liver dihydrofolate reductase in which it was shown that treatment of the enzyme with TPNH prior to electrophoresis resulted in the partial conversion of form (I) to form (II). In the present investigation, a highly purified dihydrofolate reductase from amethopterin-resistant *Lactobacillus casei* has been used to establish that form (II) differs from (I) by the inclusion of an equimolar amount of bound TPNH.

Amethopterin-resistant *L. casei* cells were grown and harvested by the procedure described elsewhere (5) for the isolation of thymidylate synthetase from this source. Dihydrofolate reductase was purified from the sonicated cells by precipitation with ammonium sulfate (35-65% fraction), filtration through BioGel P-150, and successive chromatography on hydroxylapatite and CM-Sephadex. The two principal forms of the enzyme (I and II) were resolved during the hydroxylapatite step. Following chromatography on CM-Sephadex, each form has a specific activity of about 6 μmoles of dihydrofolate reduced/min/mg protein under standard assay conditions (6). The electrophoretic mobility of forms (I) and (II) is illustrated in Fig. 1. Form (I), the slower component, is essentially homogeneous with respect to either protein or catalytic activity (gels 1 and 2). Form (II) appears to be less homogeneous (gels 3 and 4), but the catalytic activity (cf. gel 4) of the material trailing the principal band indicates that this is form (I) arising from the breakdown of (II) during electrophoresis.

Absorption spectra of (I) and (II) are shown in Fig. 2. (I) has the spectrum of a typical protein (λ_{max} at 278 mμ with a shoulder at ca. 290 mμ). In (II), however, the principal absorbance band is shifted to 274 mμ and there is an additional band at 340 mμ. The 340 mμ-absorbing material, released by heat denaturation of (II) at pH 9, was identified as TPNH by thin-layer chromatography (isobutyric acid:NH_4OH:H_2O; 66:1:33) and by its coenzymatic activity with yeast glutathione reductase. From the absorption spectrum of (II)(cf. Fig. 2) the mole ratio of TPNH to protein in (II) was calculated to be 0.9; this is based upon a value of 15,000 for the molecular weight of the

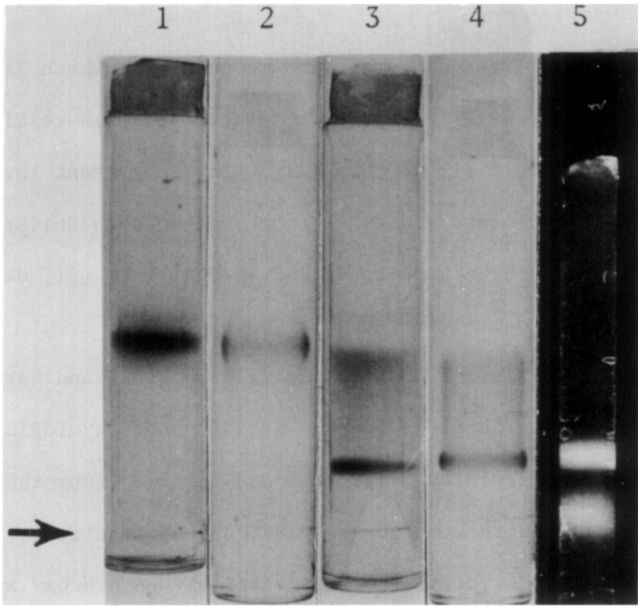

Fig. 1. Electrophoresis of forms (I) and (II) of dihydrofolate reductase from amethopterin-resistant *L. casei*. Polyacrylamide gels (0.7 x 5.5 mm) were prepared by the method of Davis (7); current, 3 mA. Arrow indicates position of the dye marker. Gels 1 and 2, form (I); gels 3-5, form (II). Gels 1 and 3 were stained for protein using Amido Black. In gels 2 and 4, enzyme activity was visualized by incubation at 37° in a mixture of 0.05 M Tris-chloride, pH 7.5, 3 x 10^{-4} M TPNH, 2 x 10^{-4} M dihydrofolate, and 1.6 x 10^{-4} M MTT-tetrazolium (1). Gel 5 (untreated after electrophoresis) was photographed under ultraviolet light (Chromato-Vue) using Polaroid Type 47 film at F16 for 15 sec with a Polaroid camera MP3 equipped with a Tiffen 2-A haze filter.

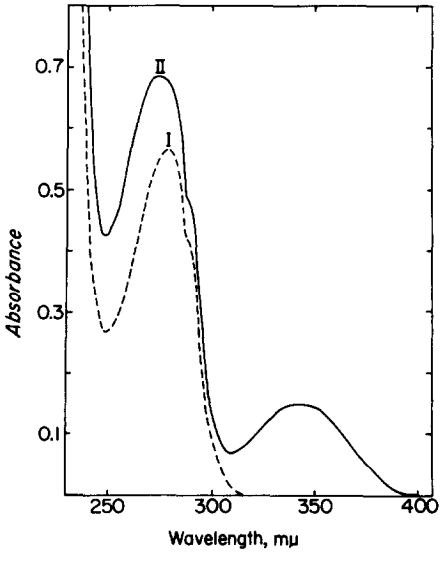

Fig. 2. Absorption spectra of (I) and (II) determined with a Cary Recording Spectrophotometer, Model 14. (I)(dashed line) and (II)(solid line) were each present at 2.6 x 10^{-5} M in 0.1 M potassium phosphate, pH 7.0.

L. casei enzyme (unpublished result of this laboratory) and assumes that the extinction coefficient at 340 mμ for the bound TPNH is the same as that for free TPNH, viz. 6.2 x 10^3 M^{-1} cm^{-1} (8).

The presence of TPNH in (II) can also be detected fluorimetrically. When (I) and (II) were subjected to electrophoresis on polyacrylamide and then examined under ultraviolet light prior to staining for protein or catalytic activity, (II) showed a blue-white fluorescence (gel 5, Fig. 1); the faster moving fluorescent band, which coincides with the dye marker, is due to free TPNH released during electrophoresis. The fluorescence spectrum of (II) is shown as curve 1 in Fig. 3. Excitation at 280 mμ results in fluorescence maxima at 340 mμ (due probably to tryptophan residues) and at 445 mμ (due to TPNH). Titration of (II) with dihydrofolate causes the 445 mμ fluorescence to decrease (and the 340 mμ fluorescence to increase), as shown by curves 2-5 in Fig. 3. If the enzyme is first treated with amethopterin, however, dihydrofolate is unable to discharge the fluorescence of the bound TPNH. This

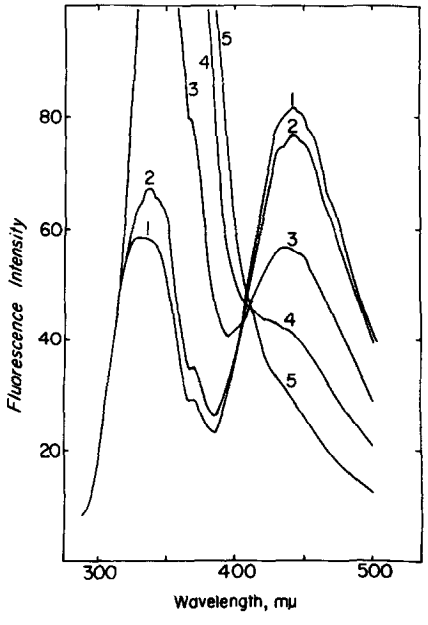

Fig. 3. Effect of dihydrofolate upon the fluorescence of (II). Fluorescence spectra were recorded using a Turner Spectro, Model 210. Excitation, 280 mμ. Volume, 3 ml. Curve 1, form (II)(1.5 x 10^{-5} M). Curves 2-5, after addition of successive increments (4.5, 22.6, 22.6, 22.6 nmoles) of dihydrofolate.

indicates that TPNH is bound at its catalytic site on the protein. In addition to being accessible to its co-substrate, dihydrofolate, the bound TPNH can also interact (although about 60-fold more slowly than free TPNH) with yeast glutathione reductase.

When form (I) is incubated at pH 8 with an excess of TPNH and then examined electrophoretically, it is converted almost completely to form (II).* Synthetic (II) is identical to the naturally-occurring form with respect to electrophoretic behavior, absorption and fluorescence spectra and 1:1 stoichiometry of pyridine nucleotide to enzyme. Pre-treatment of (I) with amethopterin does not inhibit the transformation. Exposure of (I) to DPNH, under identical conditions, does not lead to any binding of pyridine nucleotide nor to any conversion to (II). The reverse transformation, (II) → (I), is more difficult to achieve quantitatively. Treatment of natural or synthetic (II) with charcoal does, in fact, lead to the appearance of (I), but with the concommittant destruction of much of the protein. Although not as labile as form (II) of the chicken liver enzyme (4), (II) from *L. casei* loses small amounts of TPNH during electrophoretic or chromatographic procedures (cf. Fig. 1)

Although most pyridinoproteins are isolated as apoenzymes, glyceraldehyde-3-phosphate dehydrogenase, which contains bound DPN, is a well-established exception to this generalization. Recently, Jacobson *et. al.* (9) have reported that the ability of malate dehydrogenase from *Neurospora crassa* to bind varying amounts of DPN is the basis for the observed polymorphism of that enzyme. The present results establish that forms (I) and (II) of dihydrofolate reductase from *L. casei* (and probably from other sources as well) differ by virtue of one TPNH being associated with (II). This relationship can be represented by equation (2). Whether the addition of TPNH is sufficient

$$\underset{(I)}{E} + TPNH \rightleftharpoons \underset{(II)}{E - TPNH} \qquad (2)$$

* In this connection it is of interest that TPNH has been added to "stabilize" dihydrofolate reductases during purification or electrophoretic analysis (3,10,11).

to account for the increased net negative charge seen during electrophoresis, or whether TPNH binding also induces conformational changes in the protein, remains to be elucidated. It is also not yet clear whether faster-moving forms of the enzyme (e.g. form (III) in Fig. 1 of ref. 1) contain additional amounts of TPNH.

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