BBA 66521

PURIFICATION OF TETRAHYDROFOLATE DEHYDROGENASE BY AFFINITY CHROMATOGRAPHY

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(Received September 27th, 1971)

SUMMARY

Tetrahydrofolate dehydrogenase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3; formerly known as dihydrofolate reductase) from a subline of the L1210 lymphoma has been purified to apparent homogeneity with high recovery by simple steps using agarose to which the enzyme inhibitor amethopterin was coupled. A crude $(NH_4)_2SO_4$ precipitate was applied to a column of methotrexate–agarose which was washed with buffer containing NADPH to increase the binding of the enzyme to the column, and to elute non-enzyme protein. Buffer at high pH, containing dihydrofolate and high concentration of salt then eluted the enzyme together with some nucleic acid contaminants which were removed on a column of Sephadex G-75.

The enzyme tetrahydrofolate dehydrogenase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3; formerly known as dihydrofolate reductase) has been purified from several sources by a variety of conventional techniques, such as precipitation with $(NH_4)_2SO_4$ sulphate, filtration through Sephadex gels, and chromatography on hydroxylapatite or ion-exchange columns[1–4].

The high affinity of the enzyme for folate antagonists such as amethopterin (methotrexate) suggested the use of this antagonist, coupled to a solid support, to purify the enzyme. This technique of affinity chromatography has gained rapid acceptance and a number of biochemical substances have been successfully purified by this method[5–7]. Previous attempts to purify tetrahydrofolate dehydrogenase, using a methotrexate–starch complex[8] and 10-formylaminopterin–agarose[9] have also been successful.

In our method, the carboxyl group of methotrexate was coupled to aminoethyl-substituted agarose, as described by CUATRECASAS[10]. This allowed the pteroyl group to "float" far enough away from the solid matrix of the agarose, to avoid steric effects on the methotrexate–enzyme binding. In brief, we passed the enzyme solution through a column of methotrexate–agarose under those conditions of pH, substrate concentration and ionic strength where the binding of enzyme to the methotrexate was "tight" or stoichiometric[11], then washed through the contaminating proteins. The

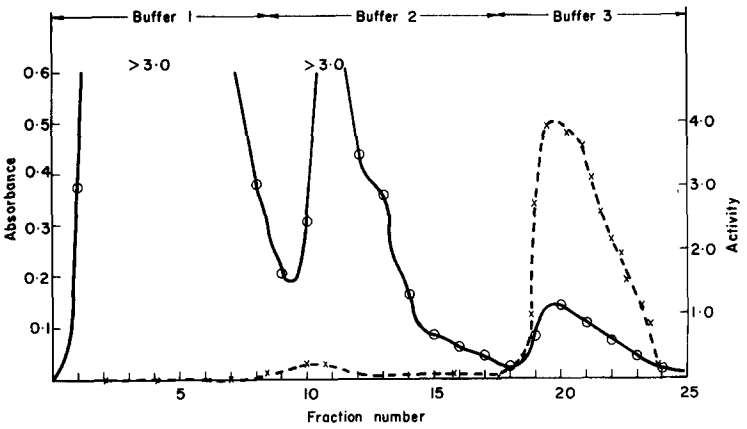

Fig. 1. Elution pattern from a column of methotrexate–agarose. The desalted $(NH_4)_2SO_4$ fraction (26 ml) was applied to a column of methotrexate–agarose and the column washed with three buffers sequentially and 4-ml fractions collected. Protein absorbance at 280 nm was measured and enzyme activity assayed. Buffer 1, 0.1 M Tris–HCl, (pH 7.5) containing $1 \cdot 10^{-5}$ M NADPH; Buffer 2, 0.2 M Tris–glycine (pH 9.5) containing 2.0 M KCl and $1 \cdot 10^{-5}$ M NADPH; Buffer 3, 0.2 M Tris–glycine (pH 9.5) containing 2.0 M KCl and $1 \cdot 10^{-5}$ dihydrofolate. ○—○, absorbance at 280 nm; ×---×, tetrahydrofolate dehydrogenase activity, μmoles/min per ml.

enzyme was eluted by changing the ionic, substrate and pH conditions, then desalted and separated from contaminating nucleic acid(s) by chromatography on Sephadex G-75.

Aminoethylagarose was prepared by the method of CUATRECASAS[10]. 10 ml of a compact slurry of the aminoethylagarose were mixed with 15 ml of 40% dimethylformamide and the pH was adjusted to 4.8 with HCl. 10 mg of methotrexate (a product of Lederle) and 5 mg of 1-ethyl-3-(dimethylaminopropyl) carbodiimide were added to the suspension which was stirred for 16 h at room temperature and then washed with 2 l of 0.1 M Tris–HCl (pH 8.5), containing 2 M KCl. A chromatography column of methotrexate–agarose (Whatman, 1 cm × 5 cm) was prepared and equilibrated with 500 ml of 0.1 M Tris–HCl (pH 7.5), containing $1 \cdot 10^{-5}$ M NADPH.

Cells were obtained from a subline (LM 4) of the L1210 lymphoma as previously described by HILLCOAT[12]. The frozen, packed cells (20 ml) were suspended in 40 ml of 0.05 M Tris–HCl (pH 7.5), stirred at 4° for 1 h and the suspension centrifuged at 15 000 rev./min for 15 min to remove cell debris. The supernatant, of specific activity 0.19 unit (μmoles of substrate reduced per min) per mg protein was treated with $(NH_4)_2SO_4$, and the fraction precipitating between 45 and 90% saturation kept. We dissolved the precipitate in 8 ml of water and added NADPH (10^{-5} M). The solution was then desalted on a Sephadex G-10 column (Whatman, 2.5 cm × 30 cm), previously equilibrated with 0.1 M Tris–HCl (pH 7.5), containing $1 \cdot 10^{-5}$ M NADPH. The active fractions were pooled and passed through the previously described methotrexate–agarose column. We washed the column with 15 ml of the equilibrating buffer and then with 40 ml of 0.2 M Tris–glycine buffer (pH 9.5) containing 2 M KCl, and 10^{-5} NADPH. This left only absorbed nucleic acid material (which was being constantly eluted) and the enzyme. The latter was then eluted by the simultaneous removal of NADPH and addition of 10^{-5} M dihydrofolate (Fig. 1). The enzymati-

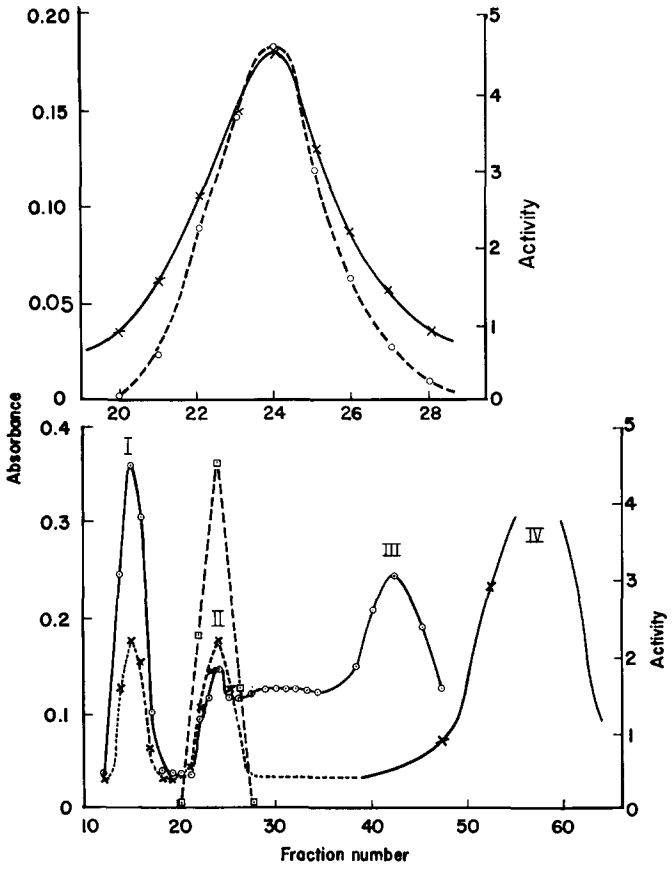

Fig. 2. Elution pattern from Sephadex G-75 column. The pooled active fractions from the methotrexate–agarose column were applied to a column of Sephadex G-75 and eluted with 0.05 M Tris-HCl buffer (pH 7.5). Fractions of 4 ml were collected. Lower graph: ○—○, absorbance at 260 nm; ×---×, absorbance at 280 nm; □---□, enzyme activity. Peak IV represents breakdown products of tetrahydrofolate. Upper graph: Peak II on a larger scale to show symmetry. ×—×, absorbance at 280 nm; ○---○, enzyme activity.

cally active fractions were pooled and applied to a Sephadex G-75 column (Whatman, 2.5 cm × 25 cm), previously equilibrated with 0.1 M Tris-HCl buffer (pH 7.8).

The enzyme was eluted from the G-75 column as a single symmetrical peak, (Fig. 2) with a specific activity of 70 units/mg of enzyme (LOWRY *et al.*[13], bovine serum albumin standard). No other protein was eluted from the G-75 column, and the enzyme was well separated from three other materials, all of which had maximum ultraviolet absorbance in the 240–260-nm region of the spectrum. The folate activity (determined by tritiated folate assay[16]) and tetrahydrofolate activity of fractions eluted from the G-75 column coincided and were in the same ratio as in the crude lysate. Using a calibrated G-100 column (method of ANDREWS[14]) we found the molecular weight of the enzyme to be 18 000 ± 1000. Acrylamide gel electrophoresis was carried out at pH 8.5 as previously described[15] and showed no contaminating protein present when 45 μg of protein was applied. The protein, folate and di-

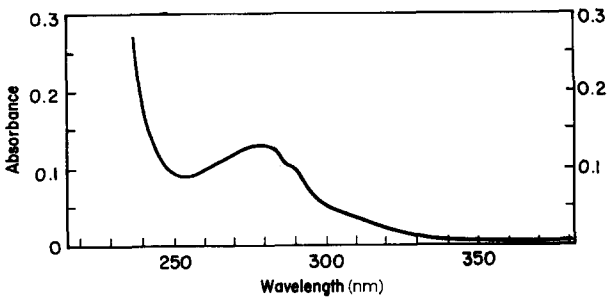

Fig. 3. Absorption spectrum of purified enzyme at a concentration of 0.04 mg/ml (calculated from titration with methotrexate, see text).

hydrofolate activity stains all coincided. The purified enzyme had the absorption spectrum of a typical protein (maximum at 278 nm with a shoulder at about 290 nm) and is shown in Fig. 3. The turnover number was determined by the method of WERKHEISER *et al.*[17] as suggested by MORRISON[18]. We added increasing amounts of enzyme to a fixed concentration of methotrexate at low ionic strength and pH (0.1 M Tris–maleate, pH 6.0) and determined the enzyme activity. The results are shown in Fig. 4. The values for the slope and horizontal intercept were obtained from a computer program[19]. The calculated turnover number was 2090 moles tetrahydrofolate per min per mole enzyme at pH 7.5 and 37°. Using this value to calculate the enzyme concentration, we observed a discrepancy between the value so obtained

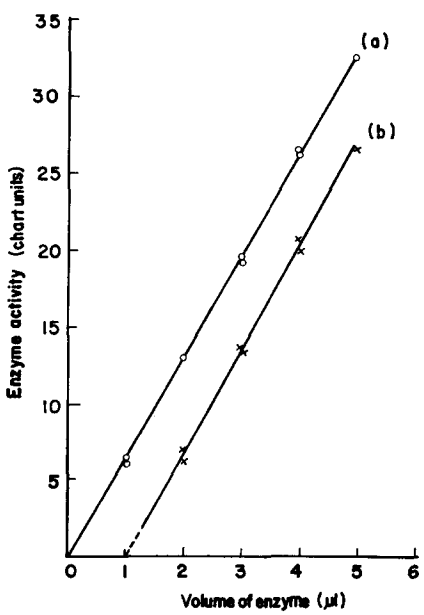

Fig. 4. Titration of enzyme with methotrexate. To a solution of $1 \cdot 10^{-8}$ M methotrexate, increasing volumes of enzyme solution were added and the reaction rate determined. Duplicate assays are shown and lines calculated by computer program (see text). ○—○, no methotrexate; ×—×, $1 \cdot 10^{-8}$ M methotrexate.

(0.031 mg/ml, spec. act. 132 units/mg) and that from the LOWRY *et al.*[13] method (0.058 mg/ml). If both were correct (0.031 mg of enzyme in 0.058 mg of protein) the enzyme preparation would be 60% pure. However, the data from chromatography on Sephadex G-75 and from gel electrophoresis indicated this not to be the case. The LOWRY *et al.*[13] method of estimating protein concentration assumes the unknown and standard (bovine serum albumin) to have a similar content of tryptophan, which may not be the case for the dehydrogenase enzyme from the L1210 cells.

TABLE I

PURIFICATION OF TETRAHYDROFOLATE DEHYDROGENASE

Step	*Activity (μmoles/min per ml)*	*Vol. (ml)*	*Total units (μmoles/min)*	*Step recovery (%)*	*Overall recovery (%)*	*Spec. Act. (μmoles/min per mg)*
1 Lysis	3.42	39	134		100	0.19
2 45–90% $(NH_4)_2SO_4$***	4.40	26	114	85	85	—
3 Agarose–methotrexate	2.24	42	94	82	70	—
4 Sephadex G-75	(not pooled)		65	69	50	70* 132**

* Protein determined by the method of LOWRY *et al.*[13].
** Protein calculated from methotrexate titration (see text).
*** Including removal of salt on Sephadex G-10 column.

If, then, we assume the enzyme preparation to be pure, and the turnover number to be 2090 moles of tetrahydrofolate produced per min per mole of enzyme, the final preparation had a specific activity of 132, and represented a 50% recovery of available enzyme from the crude lysate (Table I). It appears that the value for the specific activity based on the enzyme concentration as calculated from the methotrexate-titration data (assuming enzyme purity of 100%) is the more reliable. Further work to confirm these assumptions is being carried out.

This rapid and efficient method of purifying tetrahydrofolate dehydrogenase by affinity chromatography can be applied on a small scale as well as a large scale and should contribute to further knowledge about the properties and behaviour of this enzyme.

ACKNOWLEDGMENTS

This investigation was supported by the Medical Research Council of Canada. J.G. is post-doctoral Fellow of the Medical Research Council of Canada.

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