BBA 66505

IMPROVED PURIFICATION OF TETRAHYDROFOLATE DEHYDROGENASE FROM L1210 LEUKEMIA BY AFFINITY CHROMATOGRAPHY

PAUL L. CHELLO, ARLENE R. CASHMORE, SAMUEL A. JACOBS AND JOSEPH R. BERTINO

Department of Pharmacology, Yale University School of Medicine, New Haven, Conn. (U.S.A.)

(Received September 29th, 1971)

SUMMARY

Tetrahydrofolate dehydrogenase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3; formerly known as dihydrofolate reductase) from a high enzyme mutant of L1210 mouse leukemia was purified to homogeneity by a simple two step procedure involving pH 5.1 precipitation of inert protein, and affinity chromatography employing a methotrexate–agarose column. By raising the pH and ionic strength of the eluting buffer from 0.05 M citrate (pH 6.0) to 0.05 M Tris–HCl (pH 8.5) containing 0.1 M KCl, a peak was eluted containing pure enzyme (spec. act. 2800 μmoles/h per mg). Purity was confirmed by methotrexate titration and polyacrylamide disc electrophoresis. A second small peak containing tetrahydrofolate dehydrogenase activity was eluted by further increasing the pH of the 0.05 M Tris–HCl buffer to 9.0, and increasing the KCl concentration to 0.4 M. The material in this peak showed an absorbance at 258 nm, suggesting that a nucleotide was bound to the enzyme. The identity of this material has yet not been established.

In recent years, several macromolecules have been purified with the technique of affinity chromatography[1]. Tetrahydrofolate dehydrogenase (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3; formerly known as dihydrofolate reductase), the target enzyme of the clinically important folic acid antagonists, has been purified and characterized from several sources by conventional techniques[2–7]. The availability of many 2,4-diaminopteridine and triazine inhibitors has also led to the purification of this enzyme via affinity chromatography[8–11]. In this communication, we describe a simple, rapid purification by affinity chromatography of tetrahydrofolate dehydrogenase from a methotrexate-resistant subline of the murine L1210 lymphoma.

Beaded agarose was used as a solid support. Following a modification of the procedure of CUATRECASAS[12], agarose beads were activated by cyanogen bromide, with the subsequent attachment of ethylenediamine to form aminoethyl agarose. Methotrexate was then coupled via a carbodiimide reaction with the aminoethyl moiety.

To prepare enough gel to pack a 2.5 cm × 35 cm column, 200 ml (1 g/ml) of sepharose (Pharmacia Fine Chemicals, Inc.) were mixed with an equal volume of 0.1 M $NaHCO_3$ buffer (pH 9.0). After adjusting the pH of the slurry to 11.0 with 4 M NaOH, 26.7 g of cyanogen bromide (Eastern Chemicals Corporation) were dissolved in 33 ml of dimethylformamide (Mallinckrodt Chemical Works), and added to the agarose slurry with stirring. Since the rate of the activation reaction was temperature dependent, the reaction mixture was maintained at 20°. The pH of the reaction was maintained at 11.0–11.5 by the addition of 6 M NaOH. Activation was complete in 8–9 min, as evidenced by the stability of the pH, indicating cessation of proton release. The reaction mixture was immediately transferred to a 600-ml scintered glass filter (40–60 mesh), and washed with 5–6 l of cold distilled water, followed by 3 l of cold 0.1 M $NaHCO_3$ buffer (pH 9.0). This washed resin was resuspended in 200 ml of cold 0.1 M $NaHCO_3$ buffer (pH 9.0), and transferred to a chilled beaker; 26.6 ml of ethylenediamine (Mallinckrodt Chemical Works), previously adjusted to pH 10.0 with 12 M HCl, were added with stirring. Since the activated sepharose was highly unstable at this stage, the wash and the ethylenediamine addition were accomplished within 2 min. The reaction of the ethylenediamine with the activated sepharose was allowed to proceed with stirring at room temperature for 2 h, and then overnight at 4°.

The following morning, the aminoethyl agarose slurry was transferred to a 600-ml scintered glass filter (40–60 mesh), and washed with 2 l of cold 0.01 M NH_4OH, followed by 3 l of cold 0.1 M $NaHCO_3$ buffer (pH 9.0). The washed gel was resuspended in 200 ml of the 0.1 M $NaHCO_3$ buffer (pH 9.0), and transferred to an Erlenmeyer flask. Here, 500 mg of methotrexate (Lederle), and 620 mg of 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (Aldrich Chemical Co.) were added. After stirring for 20 h at room temperature, the methotrexate–agarose slurry was poured into a 2.5 cm × 45 cm column. The column was placed in the cold, and washed with 15 l of distilled water, followed by 2 l of 0.05 M citrate buffer (pH 6.0).

A methotrexate-resistant subline of the L1210 mouse leukemia (L1210R-512x), which was developed in this laboratory, was used as the source of tetrahydrofolate dehydrogenase. Levels of tetrahydrofolate dehydrogenase in the resistant subline are approx. 200 times higher than those found in the sensitive line. These cells may be propagated in suspension culture as well as *in vivo*. *In vitro*, the ID_{50} of methotrexate is $2.6 \cdot 10^{-7}$ M, as compared with an ID_{50} of $8.0 \cdot 10^{-9}$ M for the parent methotrexate-sensitive line.

For experimental purposes, 10^8 cells in suspension culture were treated with 10^{-6} M methotrexate for 30 min; 10^6 of these cells were then injected intraperitoneally into each of 10 male BDF_1 mice. Cells were harvested from the animals on the 7th day, and a fraction (10^6) reinjected into 75 untreated mice. After 7 days, these mice were sacrificed, and the tumor cells harvested from the peritoneal cavity by flushing with 0.9% NaCl. Packed cells were obtained by centrifugation at 8000 × *g* for 20 min in a Sorvall RC-2 Automatic Refrigerated Centrifuge. The supernatant fluid was decanted, and the cells stored at −17°.

Tetrahydrofolate dehydrogenase was extracted from the frozen cells by adding 3 vol. of cold distilled water to the cell pack, and stirring for 1 h at 4°. 1 vol. of 3.6% NaCl was added, and the lysed cell suspension centrifuged at 27 000 × *g* for 20 min in the Sorvall Centrifuge. The resultant supernatant was removed, and filtered

through cheesecloth to remove fat particles. This crude tetrahydrofolate dehydrogenase preparation had a specific activity of 4–5 μmoles/h per mg protein. A slight purification and clarification was achieved by adjusting the pH to 5.1 with 1 M HCl, and centrifuging at 27 000 × *g* for 20 min. The supernatant, after readjustment to pH 6.0, normally had a specific activity of 12–14 μmoles/h per mg protein.

TABLE I

PURIFICATION OF L121 or -512x TETRAHYDROFOLATE DEHYDROGENASE

Step	*Vol. (ml)*	*Protein (mg/ml)*	*Spec. act. (μmoles/h per mg)*	*Total activity (μmoles/h)*	*Recovery (%)*	
Crude extract	35	28.6	4.4	4410	100	
pH 5.1 supernatant	30	10.5	11.9	3750	85	
Methotrexate–Sepharose						
Peak II						
Tubes 167–179 (pooled)	65	0.010	652	4482	10.1	38.6
Tubes 180–185 (pooled)	30	0.006	2800+	591	13.4	
Tubes 186–203 (pooled)	90	0.004	1845	667	15.1	
Peak III						
Tubes 260–278 (pooled)	95	0.197	3.5	67	1.5	

The following purification with a 2.5 cm × 35 cm affinity column was repeated five times. Table I and Fig. 1 show the results of one such purification. 30 ml of pH 5.1 supernatant, having a total tetrahydrofolate dehydrogenase activity of 3750 μmoles/h, were applied to the affinity column. The column had been equilibrated with 0.05 M citrate buffer (pH 6.0), in order to insure the optimal binding of the enzyme to the methotrexate–agarose complex[13]. Beginning with 0.05 M citrate buffer (pH 6.0), 5-ml fractions were collected. Following the void volume (175 ml), a major protein peak (Peak I) appeared, which displayed no tetrahydrofolate dehydrogenase activity. After 375 ml, the elution buffer was changed to 0.05 M Tris–HCl (pH 7.0), and an additional 250 ml collected. Only small protein peaks were eluted with this buffer. The elution buffer was then changed to 0.05 M Tris–HCl (pH 8.5) containing 0.1 M KCl, and the bound enzyme eluted[13]. Following one void volume, a sharp peak of tetrahydrofolate dehydrogenase appeared (Peak II), which declined slowly. The buffer was again changed to 0.05 M Tris–HCl (pH 9.0) containing 0.4 M KCl, and an additional 225 ml collected. After collecting approximately one void volume, material with absorbance at 258 nm was eluted, which coincided with a second small peak of tetrahydrofolate dehydrogenase activity.

Using the method of WADDELL[14], the protein concentration of Fractions 166–213 was determined on a Cary Recording Spectrophotometer. Assuming pure tetrahydrofolate dehydrogenase to have a specific activity of 2800 μmoles/h per mg[6], tubes 180–185 contained pure enzyme. To verify these results, purity was determined by methotrexate titration[13,15]. This method showed a purity of 100%. The entire purification procedure, summarized in Table I, yielded a 700-fold purification of the crude extract, with a 250-fold purification resulting from the affinity chromatography.

To ascertain the homogenity of the enzyme preparation, polyacrylamide disc electrophoresis was employed. Peak fractions were concentrated to 50 μg protein/ml

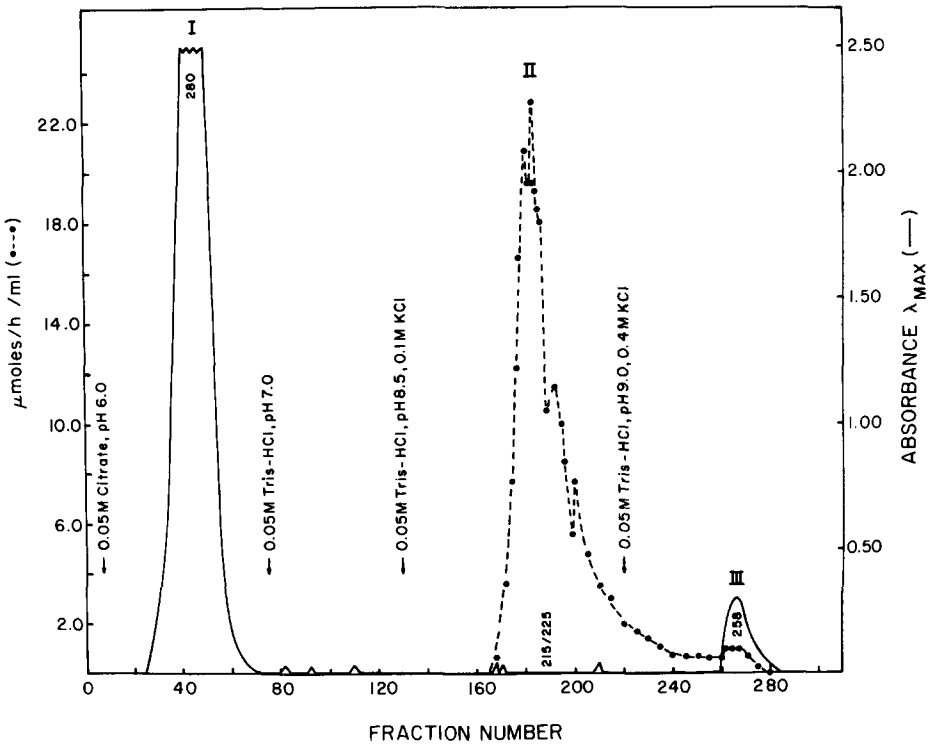

Fig. 1. Purification of L121 or -512x tetrahydrofolate dehydrogenase by affinity chromatography. Tetrahydrofolate dehydrogenase was extracted from L121 or -512x mouse leukemia cells and treated at pH 5.1 as described in the text. The enzyme preparation was adsorbed onto a 2.5 cm × 35 cm methotrexate–sepharose affinity column previously equilibrated with citrate buffer (0.05 M, pH 6.0). Three hundred 5-ml fractions were eluted with a series of four buffers: 0.05 M citrate (pH 6.0), 0.05 M Tris–HCl (pH 7.0), 0.05 M Tris–HCl (pH 8.5) containing 0.1 M KCl, and 0.05 M Tris–HCl (pH 9.0) containing 0.4 M KCl. Each fraction was assayed for tetrahydrofolate dehydrogenase activity (broken line) by method of PERKINS *et al.*[6]. The protein concentration of Fractions 1–160 was determined from the absorbance at 280 nm, as measured on a Gilford Model 2400 Spectrophotometer. The protein concentration of Fractions 160–260 was measured by the method of WADDELL[14] at 215/225 nm, using a Cary Model 15 Spectrophotometer. The λ_{max} at 258 nm shown by Tubes 260–285 was also determined with a Cary Model 15 Spectrophotometer.

by covering a dialysis bag with dry Sephadex G-200 at 4° for 4 h. 100 μl were then subjected to disc gel electrophoresis on 5% polyacrylamide according to the method of DAVIS[16], except that spacer and sample gels were omitted. Gels were stained with Amido Schwarz dye, and destained by gently shaking in frequent changes of 7% acetic acid over a 48-h period. These peak fractions contained a single distinct band of rapidly migrating protein, consistent with the known molecular weight of tetrahydrofolate dehydrogenase from L1210 mouse lymphoma, *i.e.* 20 000 (ref. 6). When Peak III, the second peak of tetrahydrofolate dehydrogenase which contained material absorbing at 258 nm, was similarly examined by disc electrophoresis, one band of protein appeared which migrated identically with the enzyme from the major reductase peak.

For processing larger quantities of crude tetrahydrofolate dehydrogenase, a 2.5 cm × 70 cm methotrexate–sepharose column adapted for upward flow was used.

A flow rate of 110 ml/h was established by placing an LKB Perplex Pump (LKB 10200; gear 9:125; tubing 1.3 mm internal diameter) between the eluant reservoir and a three-way valve (Pharmacia LV-3). In addition, the concentration of KCl in the 0.05 M Tris–HCl buffer (pH 8.5) was increased from 0.1 to 0.15 M. When a pH 5.1 treated preparation with a tetrahydrofolate dehydrogenase activity of 6200 μmoles/h was absorbed to the column, the combination of increased flow rate and increased KCl concentration produced a much sharper enzyme elution profile. This resulted in an estimated 74% recovery, of which 35% fulfilled the requirements for pure enzyme. Again two distinct peaks of tetrahydrofolate dehydrogenase were found.

Identification of the material contained in the second minor peak of tetrahydrofolate dehydrogenase which is responsible for the absorbance at 258 nm is currently underway. Although the spectra suggests a bound nucleotide, possibly a derivative of NADP, complete identification of this material has not yet been achieved.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to Mr. Frederick G. Kalfon, Technical Representative for Pharmacia Fine Chemicals, Inc., for helpful advice.

This work was supported by U.S. Public Health Service Grant CA-08010. Dr. Paul L. Chello is a Postdoctoral Fellow supported by U.S. Public Health Service Training Grant CA-05138. Dr. Joseph R. Bertino is a Career Development Awardee of the National Cancer Institute.

REFERENCES

1 P. CUATRECASAS AND C. B. ANFINSEN, *Annu. Rev. Biochem.*, 40 (1971) 259.
2 R. L. BLAKLEY AND B. M. MCDOUGALL, *J. Biol. Chem.*, 236 (1961) 1163.
3 C. K. MATHEWS AND F. M. HUENNEKENS, *J. Biol. Chem.*, 238 (1963) 3436.
4 B. T. KAUFMAN AND R. C. GARDINER, *J. Biol. Chem.*, 241 (1966) 1319.
5 S. F. ZAKREWSKI, M. T. HAKALA AND C. A. NICHOLS, *Mol. Pharmacol.*, 2 (1966) 423.
6 J. P. PERKINS, B. L. HILLCOAT AND J. R. BERTINO, *J. Biol. Chem.*, 242 (1967) 4771.
7 F. M. SIROTNAK AND J. S. SALSER, *Arch. Biochem. Biophys.*, 145 (1971) 268.
8 G. P. MELL, J. M. WHITELEY AND F. M. HUENNEKENS, *J. Biol. Chem.*, 243 (1968) 6074.
9 B. R. BAKER AND N. M. J. VERMEULEN, *J. Med. Chem.*, 18 (1970) 1143.
10 J. S. ERICKSON AND C. K. MATHEWS, *Biochem. Biophys. Res. Commun.*, 43 (1971) 1164.
11 B. T. KAUFMAN AND J. V. PIERCE, *Biochem. Biophys. Res. Commun.*, 44 (1971) 608.
12 P. CUATRECASAS, *J. Biol. Chem.*, 245 (1970) 3059.
13 J. R. BERTINO, B. A. BOOTH, A. L. BIEBER, A. R. CASHMORE AND A. C. SARTORELLI, *J. Biol. Chem.*, 239 (1964) 479.
14 W. J. WADDELL, *J. Lab. Clin. Med.*, 48 (1956) 311.
15 W. C. WERKHEISER, *J. Biol. Chem.*, 236 (1961) 888.
16 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.