PURIFICATION OF DIHYDROFOLIC REDUCTASE FROM CHICKEN LIVER BY AFFINITY CHROMATOGRAPHY

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Summary: The procedure for coupling methotrexate (4-amino-10-methylpteroylglutamic acid) to Sepharose via a six carbon chain is described. An affinity column prepared from this material quantitatively adsorbs the dihydrofolic reductase activity from a partially purified extract of chicken liver. Elution of the enzyme readily occurs with dilute K_2HPO_4 in the presence of dihydrofolate. Approximately 250-fold purification occurs by affinity chromatography yielding a preparation which appears to be homogeneous.

Dihydrofolic reductase from chicken liver has been purified by a sequence of conventional procedures using gel filtration, ammonium sulfate precipitation and chromatography on hydroxylapatite and CM-cellulose (1,2). However, due to the very small amounts of enzyme present in cells and tissues, conventional purification procedures are quite laborious if reasonable amounts of highly purified enzyme are to be isolated. On the other hand, the very strong affinity ($K_i \sim 10^{-9}$ M) between certain anti-folic compounds, such as methotrexate (4-amino-10-methyl-pteroylglutamic acid) and dihydrofolic reductase suggests that coupling of the methotrexate molecule to an insoluble matrix should yield an exceptionally efficient affinity chromatographic system. Folate compounds have been successfully coupled to proteins via a peptide linkage by a carbodiimide-promoted reaction (3,4). Extension of this procedure to the attachment of the carboxyl groups of methotrexate to aminoethyl cellulose is readily accomplished as evidenced by the formation of a highly colored cellulose derivative. However, columns prepared from this material did not adsorb significant amounts of enzyme, Mell et al., (5) coupled methotrexate to soluble aminoethyl starch and were able to purify the resulting high molecular weight enzyme-methotrexate-starch complex by means of a sequence of gel filtrations.

Recently, affinity systems based on an agarose matrix, considered by

Cuatrecasas (6) to be a nearly ideal support for affinity chromatography, has been used successfully for the purification of a number of enzymes (7-10). In some cases, effective purification depends on placing the ligand groups critical for the interaction with the macromolecule at some distance from the agarose backbone (6). These studies suggested the following route of preparation of a methotrexate column, (a) activation of a beaded agarose derivative (Sepharose) with cyanogen bromide, (b) formation of ω-amino-alkyl derivative of the activated Sepharose and (c) coupling of a carboxyl group of methotrexate to the ω-amino-alkyl-Sepharose by means of a carbodiimide-promoted reaction. Although both six and twelve carbon aliphatic diamines were used to prepare the ω-amino-alkyl derivatives of Sepharose, subsequent study revealed no significant differences in their ability to bind the chicken liver enzyme.

Sepharose 4B (25 g) was activated with cyanogen bromide (0.4 g) at pH 10.2 for 6.5 min at 25°, filtered and washed rapidly on the funnel with 300 ml of ice-cold 0.1 M $NaHCO_3$, pH 9. The damp gel was resuspended in 40 ml of cold 0.1 M $NaHCO_3$, pH 9, and 10 ml of 1.0 M 1,6-hexandiamine was added to the stirring suspension. Stirring was continued for 15 hr at 2°. The suspension was filtered, washed with about 100 ml each of 0.1 M $NaHCO_3$, pH 9, water, 1 N HCl, water, 1 N NaOH and water. The resulting ω-aminohexyl derivative of Sepharose was reacted with 230 mg methotrexate (0.5 mmole) and 456 mg (2 mmole) of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide at 25° for 1.5 hr while stirring and maintaining the pH at 6.4 with 1 N HCl. The bright yellow-orange gel was washed successively with large volumes of 1 M K_2HPO_4, water, 1 M KH_2PO_4 and water until the resulting filtrates were colorless. The final product was stored as an aqueous suspension in the cold and protected from light.

The effectiveness of the resulting adsorbent was demonstrated by passage of a small amount of highly purified chicken liver dihydrofolic reductase through a small column (∿1 ml bed volume). Quantitative adsorption occurred at pH 5.6. Subsequent investigation demonstrated that the enzyme activity could be readily eluted by 0.05 M K_2HPO_4 containing a small amount of dihydro-

folate ($\sim$1 x 10^{-5} M). However, in the absence of dihydrofolate in the elution buffer, considerable tailing of the activity peak occurred.

Attempts to quantitate the capacity of such a column did not prove practical since a definitive "break through" of activity was not obtained with the amount of purified enzyme available. A very gradual increase in activity in the effluent was the only indication that the enzyme was no longer being effectively adsorbed. These observations, as well as other studies, suggest that a multiplicity of sites of varying affinity for the enzyme are most probably present.

Attempts to adsorb the enzyme activity directly from a crude chicken liver homogenate was not successful. Very little of the activity was adsorbed as compared with the minimum potential capacity of the column as determined with highly purified enzyme. However, after preliminary fractionation of the crude material by gel filtration on a Sephadex G-75 column, the predicted amount of activity was now quantitatively adsorbed by the methotrexate column. These studies suggested that unknown materials of higher molecular weight are present in the crude extract which interfere with the binding of the enzyme. In addition, subsequent studies also revealed that substances are adsorbed to the column far more strongly than dihydrofolic reductase since its elution required 6 M guanidine hydrochloride.

Figure 1 illustrates the affinity chromatography of chicken liver dihydrofolic reductase activity from a Sephadex G-75 eluate. Following extensive washing with the starting buffer (0.01 M potassium phosphate, pH 5.6) increasing the phosphate concentration to 0.5 M resulted in the elution of a small peak of inactive material. After rewashing to the initial buffer concentration the elution of the enzyme is initiated by allowing 2 ml of 0.05 M K_2HPO_4 containing approximately 2 mg of dihydrofolate to absorb into the column bed and following this with 0.05 M K_2HPO_4. The activity is immediately eluted as a relatively sharp peak. Figure 1 also illustrates that increasing the concentration of the K_2HPO_4 to 1 M elutes something which is bound to the column more tightly than the enzyme.

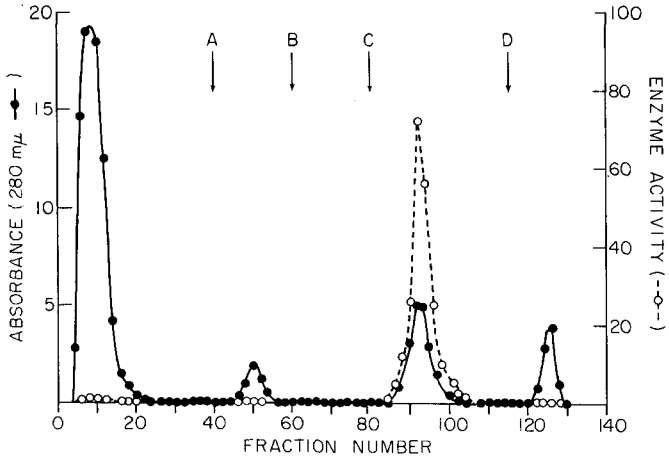

Figure 1. Adsorption and elution of chicken liver dihydrofolic reductase on a Methotrexate-Sepharose (4B) column. The conditions are essentially those described in the text. The extract (20 ml) was applied to the column (12 x 100 mm) and washed in with 0.01 M phosphate buffer, pH 5.6. At the designated intervals the following reagents were introduced into the column: (A) 0.5 M phosphate buffer, pH 5.6, (B) 0.01 M phosphate buffer, pH 5.6, (C) 1 ml 0.05 M K_2HPO_4 containing 2 mg dihydrofolate followed with 0.05 M K_2HPO_4 and (D) 1 M K_2HPO_4. Enzymatic activity was measured by the spectrophotometric procedure of Kaufman and Gardiner (1).

In order to remove the large amount of dihydrofolate in the sample, the combined active fractions were passed through Sephadex G-25 column. Examination of the spectrum of the resulting preparation (shoulder between 310 and 340 mμ) suggests that considerable amounts of dihydrofolate must be bound to the enzyme at this stage. In view of the observation that passage of a methotrexate-inhibited enzyme through a hydroxylapatite column displaced the inhibitor from the enzyme (11), the combined active fractions from the G-25 column were absorbed and eluted from a small hydroxylapatite column in order to similarly displace the bound dihydrofolate. The resulting preparation now exhibited a typical protein spectrum with a specific activity of approximately 10 μmoles of dihydrofolate reduced per min per mg of protein. This corresponds to a purification of about 240-fold via the affinity column with an overall recovery at this step of 75-100%.

Table I summarizes the purification of chicken liver dihydrofolic reduc-

TABLE I. Purification of chicken liver dihydrofolic reductase

	Volume ml.	Activity units/ml	Protein mg/ml	Total Activity units	Specific Activity units/mg protein ($x10^3$)	Yield %
1. Supernatant from homogenate	720	0.10	67	72	1.5	100
2. Protamine Sulfate extract	810	0.09	39	72	2.3	100
3. 35 to 85% Ammonium** sulfate and Sephadex G-75	600	0.08	3	48	27.0	67
4. 50 to 80% Ammonium** Sulfate	120	0.30	7.5	36	40.0	50
5. (a) Methotrexate Column	35	1.0	*	35	-	49
(b) 0 to 85% Ammonium** sulfate	2	17.5	*	35	-	
(c) Sephadex G-25	8	4.1	*	33	-	
(d) Hydroxylapatite	15	2.1	0.22	32	9,600	45

* Protein cannot be determined due to the presence of dihydrofolate.

** % of saturation at 4°.

tase based on the previously discussed observations. The initial steps in this procedure were essentially as previously described (1) except 450 grams of chicken liver were used as the starting material. The 50 to 80% ammonium sulfate fraction after the first Sephadex G-75 step is dissolved in about 120 ml of 0.01 M potassium phosphate buffer, pH 5.6, and allowed to percolate through the methotrexate column (20 x 100 mm) at a flowrate of not more than 0.5 ml/min. Washing the column and elution of activity was carried out as described for figure 1.

The activity in the combined eluates are precipitated with ammonium sulfate, dissolved in a minimum volume (2cc) of 0.01 M potassium buffer, pH 6.8, and desalted by passage through a Sephadex G-25 column (25 x 250 mm) using 0.01 M potassium phosphate buffer, pH 6.8. The active fractions are

immediately adsorbed into a hydroxylapatite column (25 x 100 mm, equilibrated with 0.01 M phosphate buffer, pH 6.8) and exhaustively washed with the starting buffer. The activity is eluted by means of a linear gradient of increasing concentrations of potassium phosphate buffer, pH 6.8, from 0.01 to 0.4 M .

This represents an overall purification of approximately 6500-fold with a total recovery of 45 to 50%, and is to be compared with the previously repeated procedure (1) where the overall recovery was less than 10%. The product exhibits a single protein band in both standard polyacrylamide gel electrophoresis and electrofocusing.

Current studies are concerned with the utilization of this affinity system for the purification of dihydrofolic reductase from yeast and bacteria. Preliminary studies have demonstrated that adsorption and elution occurs quite readily. However, again methotrexate-sepharose adsorbs large amounts of unknown high molecular weight proteins from the crude extracts.

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