A General Method for Purification of Deoxycytidine Kinase

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Deoxycytidine kinase from a variety of animal tissues binds to Cibacron Blue 3G-A coupled to Sepharose CL-6B. The enzyme can be selectively eluted with ATP to yield single-step purifications of 17–89-fold. The affinity adsorbent is re-usable.

Affinity chromatography on Cibacron Blue 3G-A coupled to a matrix has enabled the purification of a number of enzymes containing a dinucleotide fold (Thompson *et al.*, 1975) which interacts with the dye. Specificity is achieved by selective elution procedures.

Deoxycytidine kinase (EC 2.7.1.74), a salvage enzyme involved in the biosynthesis of deoxyribonucleotides for incorporation into DNA, is found mainly in tissues in which cell division is occurring, e.g. lymphoid tissues or tumours (Durham & Ives, 1969). The enzyme has an important role in the activation of the chemotherapeutic agents 1- β -D-arabinofuranosylcytosine (Goodell *et al.*, 1971) and thiodeoxyguanosine (Nakai & Le Page, 1972), the former being widely used in the treatment of acute leukaemia. In order to ascertain whether the properties of this enzyme are altered in the neoplastic state, it is desirable to obtain the purified enzymes from normal and tumour tissues.

In this paper we describe the partial purification of deoxycytidine kinase from normal tissues or tumours from several animal sources, using affinity chromatography on Cibacron Blue coupled to Sepharose. Specific elution is achieved by using the donor substrate ATP and elevated pH. Enzymes obtained in this way are sufficiently purified to enable comparison of their kinetic properties.

Materials and Methods

Calf thymus was obtained from freshly slaughtered animals. Pig tissues were obtained from control and tumour-bearing animals of an experimental herd. Lymphocytes were obtained from the peripheral blood of a patient suffering from chronic lymphocytic leukaemia and were separated from other cells by discontinuous density-gradient centrifugation (Boyum, 1968).

Samples containing deoxycytidine kinase in a form suitable for application to the affinity column were obtained by homogenization of the tissues and $(\text{NH}_4)_2\text{SO}_4$ precipitation (Durham & Ives, 1970). $(\text{NH}_4)_2\text{SO}_4$ fractions used were calf thymus (40–62.5%), pig lymph node tumour and control (40–65%) and pig lymphoma liver (40–62.5%).

Lymphocytes (6×10^8 cells/ml) were homogenized in 50 mM-Tris/HCl, pH 7.4, containing 5 mM-MgCl₂ and 5 mM-2-mercaptoethanol, in a Teflon/glass homogenizer (0.1 mm all round clearance) until no whole cells were visible by microscopy. The homogenate was centrifuged at 40000g for 20 min and the supernatant was applied directly to an affinity column. All purification procedures were carried out at 4°C.

The affinity adsorbent was prepared by the method of Bohme *et al.* (1972), with Sepharose CL-6B as the supporting matrix. By the method of Chambers (1977) the amount of dye bound was found to be 4.4 $\mu\text{mol/ml}$ bed volume of affinity adsorbent. Sepharose CL-6B was purchased from Pharmacia, Uppsala, Sweden. Cibacron Blue 3G-A was a gift from Ciba-Geigy (U.K.), Dyestuffs Division, Paisley, Scotland, U.K. Chromatography was accomplished in columns (14 mm $\times$ 65 mm or 9 mm $\times$ 48 mm) of affinity adsorbent. Disodium ATP (grade II) was obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Enzyme samples, at a protein concentration not exceeding 3 mg/ml, were applied to columns equilibrated with 50 mM-Tris/HCl, pH 7.4, containing 5 mM-MgCl₂ and 5 mM-2-mercaptoethanol, and the columns were washed with 5 column volumes of this buffer. Columns were then washed with 6 column volumes of buffer containing 0.4 M-KCl, followed by 2 column volumes of 50 mM-Tris/HCl, pH 9, containing 5 mM-MgCl₂ and 5 mM-2-mercaptoethanol. Elution of the enzyme was accomplished with 50 mM-Tris/HCl, pH 9, containing 5 mM-2-mercaptoethanol and 10 mM-ATP. Application of the sample was carried out at a flow rate of 1 column volume/h, and washing and elution procedures were at 2 column volumes/h. After elution was complete, columns were regenerated by washing with 3 column volumes of 50 mM-Tris/HCl, pH 9, containing 5 mM-2-mercaptoethanol and 2 M-NaCl. Before re-use columns were equilibrated to pH 7.4.

Deoxycytidine kinase activity was measured as described by Ives *et al.* (1969). One unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 nmol of product in 30 min at 37°C.

Protein was determined colorimetrically by the method of Bramhall *et al.* (1969). Bovine serum albumin (fraction V) obtained from Sigma was used as a standard.

Results and Discussion

Affinity columns of Cibacron Blue coupled to Sepharose CL-6B achieve specificity by variation of the elution conditions for different enzymes. Use has been made of the donor substrate ATP for eluting deoxycytidine kinase from the adsorbent. Columns of Sepharose CL-6B alone failed to bind any deoxycytidine kinase, indicating that there is an interaction between the enzyme and the dye.

Table 1 shows the loading characteristics of the columns. The enzyme requires to be applied at near neutral pH and in a buffer of low ionic strength. Ethanediol, which is frequently used for the stabilization of the activity of purified deoxycytidine kinase, prevents the binding of the enzyme to the adsorbent. The adsorbent has similar capacities for the enzyme from different tissues. However, a striking difference in capacity is observed between the enzymes from lymph nodes of control and lymphoma-bearing pigs. Although these enzymes co-electrophorese on polyacrylamide gels, the tumour enzyme exhibits a higher affinity for cytosine arabinoside than does the normal enzyme (A. Baxter, L. M. Currie & J. P. Durham, unpublished observations).

Fig. 1 shows the elution profile obtained for the calf thymus deoxycytidine kinase on a 10ml column. The general features are repeated for the enzyme from the other tissues. Saturation of the column is achieved when the enzyme is eluted with the column equilibrating buffer. The KCl wash elutes large amounts of protein having no deoxycytidine kinase activity. Haemoglobin present in samples is bound to the adsorbent and is only eluted during the high-salt wash. The affinity of haemoglobin for Cibacron Blue leads to interference with the binding of the enzyme

in samples such as liver and spleen. In such cases the KCl wash elutes small constant amounts of activity.

Elution patterns for deoxycytidine kinase using different eluting conditions are shown in Table 2. The enzyme can be eluted by using the phosphate donor substrate ATP at elevated pH. Elevated pH alone does not elute the enzyme. Similarly 10mM-ATP at pH 7.4 gives 60% of the enzyme activity recoverable at pH 9.0. The enzyme has a requirement for Mg^{2+} ions, but elution at elevated pH in the presence of 12mM- $MgCl_2$ and 10mM-ATP consistently gives a lower recovery and purification of the enzyme. Of equal importance is the observation that, in the absence of Mg^{2+} ions, 70–80% of the recoverable enzyme activity is obtained in the second column volume of eluate. In the presence of Mg^{2+} ions, the enzyme activity is spread over the second and third eluted column volumes, leading to a more dilute and less stable enzyme.

Recoveries of enzyme activity and purifications are shown in Table 1. The remaining activity is not accounted for, although it is known that the purified enzyme is relatively unstable. Inclusion of ethanediol in the column buffers is impractical as it interferes with the enzyme binding. Moreover, ethanediol in the eluting buffer markedly decreases the purification without increasing recovery (Table 2). The preparations obtained contain only a single species phosphorylating deoxycytidine as indicated by polyacrylamide-gel electrophoresis by the method of Kit *et al.* (1973).

Purification of enzymes by affinity chromatography is especially useful when dealing with small amounts of sample as in tumour biopsies or peripheral blood samples. Deoxycytidine kinase may be important therapeutically in some tumours and a rapid method of purification is desirable to obtain enzyme samples suitable for molecular and kinetic characterization. Cibacron Blue coupled to Sepharose appears to be a suitable affinity adsorbent for the deoxycytidine kinases from several tissue sources. A further

Table 1. *Loading characteristics, recovery and purification of deoxycytidine kinases on affinity columns*

Samples, prepared from various animal tissues, containing deoxycytidine kinase were loaded on to columns of affinity adsorbent at a protein concentration not exceeding 3mg/ml. The size of sample saturating a column was dictated by the enzyme activity which could be added, generally about 4 units/ml bed volume. Recovery of enzyme activity was determined as a percentage of the activity applied and purification factors were obtained after determination of the specific activities of the applied and eluted samples.

Enzyme source	Activity added (units/ml bed volume)	Protein added (mg/ml bed volume)	Recovery of enzyme activity (%)	Purification (fold)
Calf thymus	4.6	1.8	51	89
Pig lymph node (lymphoma)	6.8	6.0	44	39
Pig lymph node (control)	4.0	3.2	55	49
Pig liver (lymphoma)	3.8	2.0	39	17
Human leukaemic lymphocytes	4.1	0.3	37	39

Fig. 1. *Cibacron Blue-Sepharose column affinity chromatography of calf thymus deoxycytidine kinase*
A sample, containing 46 units of deoxycytidine kinase and 18 mg of protein in a total volume of 7 ml, was loaded on to an affinity column (14 mm $\times$ 65 mm). Enzyme activity (—) and protein (----) were measured in the column effluent. Unbound enzyme is eluted with the equilibrating buffer (1–50 ml). The bulk of the protein is eluted in a high-salt fraction (51–110 ml) and 80% of the recoverable activity is eluted in a single fraction (141–150 ml).

Table 2. *Relative recovery and purification of deoxycytidine kinase on affinity chromatography with different elution conditions*

Samples, prepared from calf thymus, containing deoxycytidine kinase were applied to affinity columns (9 mm $\times$ 48 mm) and washed as described in the Materials and Methods section. The efficiencies of different elution conditions (namely additions to 50 mM-Tris/HCl/5 mM-2-mercaptoethanol) were compared with that of the standard elution procedure (50 mM-Tris/HCl, pH 9, containing 5 mM-2-mercaptoethanol and 10 mM-ATP). Relative recoveries are expressed as a percentage of the enzyme activity eluted under standard conditions. Comparative purifications are also shown. Standard recovery and purification of the calf thymus enzyme are shown in Table 1.

Elution conditions	Recoverable activity (%)	Relative purification
10 mM-ATP, pH 9	100	1
10 mM-ATP, 12 mM-MgCl ₂ , pH 9	90	0.92
pH 9	0	—
12 mM-MgCl ₂ , pH 9	0	—
10 mM-ATP, pH 7.4	60	1.2
10 mM-ATP, 10% ethanediol, pH 9	100	0.3

advantage of this system is that the affinity adsorbent is re-usable without a decrease in its capacity for the enzyme.

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