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AFFINITY PURIFICATION OF HUMAN DEOXYCYTIDINE KINASE: AVOIDANCE OF STRUCTURAL AND KINETIC ARTIFACTS ARISING FROM LIMITED PROTEOLYSIS

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Homogeneous deoxycytidine kinase has been isolated from leukemic human T-lymphoblasts by affinity chromatography based on a multisubstrate analog, deoxycytidine 5'-adenosine 5'''-P^1,P^4-tetraphosphate (dCp_4A). Chromatography of extract treated with protease inhibitors yielded a monomeric polypeptide, inasmuch as the M_r of the native protein, 59,300, is comparable to the value of 52,000 from sodium dodecyl sulfate polyacrylamide gel electrophoresis. The isoelectric pH was 6.1. But, enzyme isolated without protease inhibitors exhibited two fragments of M_r = 30,000 and 33,000, suggesting that proteolytic cleavage of the parental polypeptide had occurred during affinity chromatography. Both the parental and proteolyzed enzymes phosphorylated deoxyadenosine and deoxyguanosine, as well as deoxycytidine. However, the proteolyzed enzyme had an increased apparent K_m for deoxycytidine. In consequence of this, a mixture of the two forms produced bimodal kinetic plots, whereas linear kinetics were displayed by each form alone.

The identification and characterization of the enzymes responsible for phosphorylating deoxyadenosine, deoxyguanosine and deoxycytidine in human cells continues to be a challenging and elusive problem. While mutant cells lacking these activities can continue to grow, their resistance to chemotherapeutic nucleosides reveals that one or more of these activities plays an important practical role in the activation of these drugs (1-3). These kinase activities also contribute to the cytotoxic effects of purine deoxynucleosides in cases of impaired catabolism of dAdo (4) or dGuo (5), resulting in immunodeficiency diseases.

Each of the dCyd or purine deoxynucleoside kinases partially purified from the cytosol of human cells has been able to phosphorylate more than one deoxynucleoside substrate, but in varying ratios, and with various K_m values (6-8). It is also unclear how many multispecific kinases are expressed in human cytosol. For example, two fractions having all three activities, but with a different set of apparent K_m values, have been obtained from T-lymphoblasts, in contrast to a single multispecific dCyd kinase from B-lymphoblasts (6). Osborne has reported a dAdo-dGuo kinase from mutant CCRF-CEM T-cells lacking both dCyd and Ado kinase activities (9), and Hurley et al. (7) have identified three fractions from human placental extract as dGuo-dCyd kinase, dCyd-dAdo kinase and Ado-dAdo kinase. Sarup and Fridland purified human T-lymphoblast and myeloblast kinases more extensively (8), and Bohman and Eriksson have recently isolated homogeneous dCyd kinase from human leukemic spleen (10). In each case, the dCyd kinase also phosphorylated dAdo and dGuo. Progress towards a clear understanding of the kinetic and regulatory mechanisms affecting human deoxynucleoside kinases has been impeded by the bi-modal or nonlinear kinetic patterns often obtained when nucleoside or ATP is varied (2, 8, 11). Such results mimic those seen earlier with animal cell preparations (12, 13). Typically, double-reciprocal plots have yielded two slopes, from which two K_m values have been derived. It has not been clear whether this phenomenon is due to cooperative behavior, or to a mixture of enzyme forms. In this paper we will show that limited proteolysis, which can occur during purification of the human enzyme, gives rise to a second active form having kinetic properties which differ from those of the parental enzyme and which may contribute to non-linear kinetic patterns.

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EXPERIMENTAL PROCEDURES

Materials--Human leukemic lymphocytes, obtained by leukopheresis, were provided by the Tumor Procurement Service of the OSU Comprehensive Cancer Center. The majority of the cells in a bone-marrow biopsy were primitive T-lymphoblasts, as characterized by E-rosette formation and cell-surface antigens, leading to a diagnosis of acute lymphoblastic leukemia (ALL). The bisubstrate affinity column, dCp_4A-Agarose, was synthesized as described previously (14), obtaining 2.6 umol of substituent per ml of gel.

Enzyme preparation and assay--After the lymphocytes were washed and freed of red cells by hypotonic lysis they were disrupted by brief sonication in Seligmann balanced salt solution also containing 0.05 % EDTA and 2 mM dithioerythritol. The 20,000 x g supernatant fraction was quick-frozen and stored at -80 °C. Preliminary to purification of the enzyme, nucleic acids were precipitated with streptomycin sulfate (1 g/g protein, pH 7.0). The supernatant solution was fractionated with ammonium sulfate; the protein precipitating between 30% and 63% saturation was dissolved in equilibration buffer (EB)--20 mM Tris buffer (pH 7.5) containing 20% glycerol and 2 mM dithioerythritol. Kinases were routinely assayed as described previously (15) at pH 7.5 and 37 °C, in 5% glycerol, aided by a disk-washing device (16). One unit of enzyme activity is defined as that amount of enzyme which catalyzes the formation of 1 nmol of deoxynucleotide per minute. Protein concentrations were measured by the Bradford method (17).

Affinity chromatography--A 3-ml column of dCp_4A-Sepharose was equilibrated with EB. Several protease inhibitors were added to the sample before it was passed onto the affinity column, but the column itself was not equilibrated with these inhibitors, because they caused a larger proportion of the activity to run through the column. The final protease inhibitor concentrations in the sample were: 1,10-phenanthroline (5 mM), benzamidine (1 mM) and diphenylcarbamyl chloride (0.1 mM). After adding the sample at 4° over a three-hour period, the column was washed with 10 vol of equilibration buffer (EB), followed by 10 vol of the same plus 0.3 mM ATP and 0.5 M NaCl to remove any nonspecifically-bound proteins. Then, before eluting the enzyme, the ATP and salt were removed by washing again with 5 vol of EB. Finally, flow through the column was reversed, and the dCyd kinase was eluted by adding 0.3 mM dCTP in EB to the column and allowing it to stand overnight before resuming flow and collecting the eluate. (Pronounced tailing of the elution peak occurred unless the column was allowed to equilibrate overnight with the dCTP, presumably because of an unusually slow off-rate for the ligand-bound enzyme.) Mg^{2+} ion was not required either for retention or elution of enzyme.

Electrophoresis and isoelectric focusing--Discontinuous non-denaturing polyacrylamide gel electrophoresis was run according to the procedure of Laemmli (18), but without SDS, using a Bio-Rad mini slab-cell at 4°, and staining with Coomassie Blue or silver (19). Enzyme activies were assayed in unstained parallel channels cut into 2 mm slices as described previously (20). Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (18) was run at room temperature, with 4% stacking gel and 12% separating gel. Horizontal isoelectric focusing was performed as described by Righetti (21), using a thermoelectrically-cooled Hoefer 950 Isobox electrofocusing unit at 4°, developed at a maximum of 2000 V constant power. Either 5% polyacrylamide or 1% agarose gel was cast on the hydrophilic surface of GelBond film, and contained 2% Ampholite, pH 3-10. A channel was cut into 2 mm slices which were soaked in water, and the pH was measured. Enzyme activity was located in gel slices assayed as above.

Molecular weights--The relative molecular mass (M_r) of the unproteolyzed enzyme was determined by sedimentation equilibrium at 4° in a Beckman Airfuge by the method of Pollet (22), modified as described previously (23). Either bovine serum albumin or Dextran T-40 was added (5 mg/ml) to the dCyd kinase samples to stabilize the density gradients, and glycerol was added to standard tubes to equal its concentration (6.7%) in the diluted enzyme samples. Ten successive 10-ul samples withdrawn from the meniscus of each tube were assayed for enzyme activity or protein content. Molecular weights were also estimated by the observing the effect of polyacrylamide gel concentration (8, 10, 12 and 15%) on electrophoretic mobilities of native proteins (24). Standard proteins included ß-lactoglobulin, carbonic anhydrase, ovalbumin and bovine serum albumin.

RESULTS

Purification of deoxycytidine kinase--Depending on the sample load applied to the dCp_4A-Sepharose affinity column, from 13 to 60% of the dCyd kinase activity applied was recovered in the fraction eluted by its end-product, dCTP, while the amount of activity in the runthrough fraction also increased in proportion to the amount of sample applied. A typical application of 750 mg of protein resulted in recovery of 16-20% of the activity, with a specific activity of 21 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} (corrected for inhibition by dCTP). This activity was eluted biospecifically in 1-2 column volumes of dCTP (after overnight equilibration), whereas 10 column-volumes of ATP and salt did not displace it from the column. Therefore, enzyme interaction with the column was probably not due to ion-exchange or nonspecific nucleotide interactions with the phosphate-donor site of the enzyme. A mixture of dCyd and ATP was considerably less effective than dCTP as an eluent. Columns were used repeatedly over a period of a year or more, with only a gradual deterioration of performance being noted.

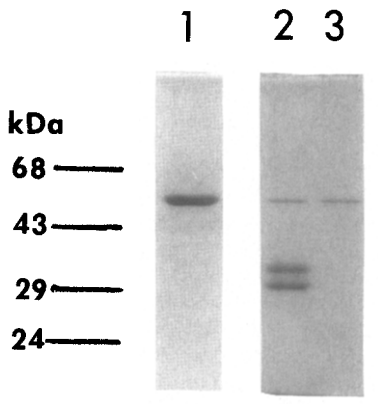

Figure 1. SDS-polyacrylamide gel electrophoresis of dCyd kinase purified with and without protease inhibitors. Lane 1, kinase purified with protease inhibitors; Lane 2, kinase purified without protease inhibitors; Lane 3, diluted kinase purified with protease inhibitors. Arrows denote R_fs of molecular weight marker proteins (Sigma).

Enzyme purity and subunit molecular weight--Judging from the single band obtained with the affinity-purified enzyme in SDS-polyacrylamide gel electrophoresis (Figure 1, lane 1), this preparation was virtually homogeneous, with an M_r of 52,000 ± 2000 for the denatured polypeptide. Only one band of protein was observed upon electrophoresis in nondenaturing gels as well. Assurance that the stained band was, in fact, the enzyme was provided by activity assays in gel slices from a parallel channel. A series of such assays revealed the congruent migration of activity and the protein band at four concentrations of acrylamide monomer. Had the protein bearing the activity and the protein yielding the stained band been different molecular species, they could have been expected to diverge as the gel concentration was changed (24). Since they did not, and since only one band was detected by either procedure, we can conclude that the enzyme was practically homogeneous.

Molecular weight of native enzyme--Sedimentation equilibrium determinations (22), carried out with two batches of dCyd kinase prepared in the presence of protease inhibitors, yielded an average molecular weight of 59,300 ± 3700 for the native protein, assuming a partial specific volume of 0.725. An identical value was obtained from the mobilities of the enzyme activity after electrophoresis in polyacrylamide gels of various concentrations (24), even though the latter determination is affected by the shape as well as the mass of the molecule. Although the M_r estimated for the native enzyme is somewhat larger than the 52,000 value determined for the denatured polypeptide, it seems clear that this enzyme consists of a single polypeptide chain.

Isoelectric pH--Isoelectric focusing of the pure enzyme on a flat-bed polyacrylamide gel system yielded a single peak of activity centered on a pI value of 6.1 ± 0.1. The presence of a slight shoulder on the high-pH side of the peak suggests that a minor polymorphic form of this protein may be present.

Specificity--The pure protein retained the ability to phosphorylate dAdo and dGuo, but dCyd is clearly the preferred substrate in terms of its apparent K_m and efficiency ratio, as may be seen in Table I. Potential phosphate

Table I
SUBSTRATE SPECIFICITY OF HUMAN DEOXYCYTIDINE KINASE

Nucleoside	K_m(app) (uM)	V_{max} (nmol·min^{-1}·mg^{-1})	Efficiency Ratio (V_{max}/K_m)*
dCyd	3.3	21	6400
dAdo	890	41	46
dGuo	640	28	44

*K_m expressed in mM units.

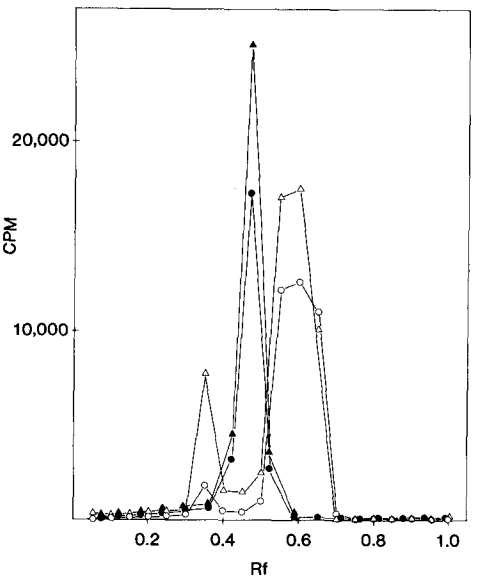

Figure 2. Non-denaturing polyacrylamide gel electrophoresis of parental and proteolyzed dCyd and dAdo kinase. Assays were performed on gel slices from an unstained adjacent channel. The acrylamide monomer concentration was 12%.

●, parental dCyd kinase; ▲, parental dAdo kinase; o, proteolyzed dCyd kinase; Δ, proteolyzed dAdo kinase.

donors were also compared, each at 10 mM (with 12 mM $MgCl_2$). Relative rates were: ATP, 100; GTP, 61; dGTP, 59; dTTP, 37; CTP, 24; dATP, 23; UTP, 6; and dCTP, 0. These nucleotides were used straight from the bottle without any repurification, so it is possible that the relatively low activity seen with UTP could have been due to an inhibitory level of UDP (12). These patterns of specificity are similar to those seen with other human deoxycytidine kinase preparations (8, 10), and it seems highly likely that the purine deoxynucleoside kinase activities are inseparably associated with the dCyd kinase protein.

Conditional proteolytic modification during purification--Enzyme purified in the absence of protease inhibitors showed evidence of substantial proteolysis during affinity chromatography, with the parental (presumably unproteolyzed) 52 kilodalton band giving place to bands of 30 kDa and 33 kDa in SDS electrophoretic gels. Figure 1 (Lane 2) shows very clearly that nearly all of the parental protein in the eluate was replaced by two smaller fragments staining with roughly equal intensity. Lane 3 contains diluted parental protein, which is identical in size to the residual minor band of the proteolyzed fraction. The discrete character of the proteolyzed bands and the complete absence of smearing below the parental band make it appear likely that the 30 and 33 kDa bands are the result of a discrete cleavage event, rather than being due to progressive proteolysis of the 52 kDa polypeptide. No smaller fragment was detectable, either by Coomassie Blue or silver staining.

The proteolyzed enzyme retained activity despite altered molecular properties, but that activity was labile and was soon lost. The electrophoretic R_f of the proteolyzed enzyme activity in nondenaturing gels shifted to about 0.60 (Figure 2), compared with 0.47 for the native enzyme run under the same conditions. A less active minor component was also seen at R_f 0.35, corresponding to a barely-detectable band on the stained gel (not shown). The increased R_f after proteolysis is presumed to be due to a reduced molecular mass, but activity losses prevented the completion of molecular weight determinations. Figure 2 also shows that, for both the parental and proteolyzed enzyme, dAdo kinase activity is exactly congruent with dCyd kinase activity, as would be expected if both substrates were phosphorylated by the same protein in each fraction. However, it should not necessarily be inferred from this figure that the ratio of dCyd/dAdo kinase is constant; since the assay of gel slices extended over a number of hours, differential loss of enzyme activities could have occurred, so the radioactivities plotted should be viewed in

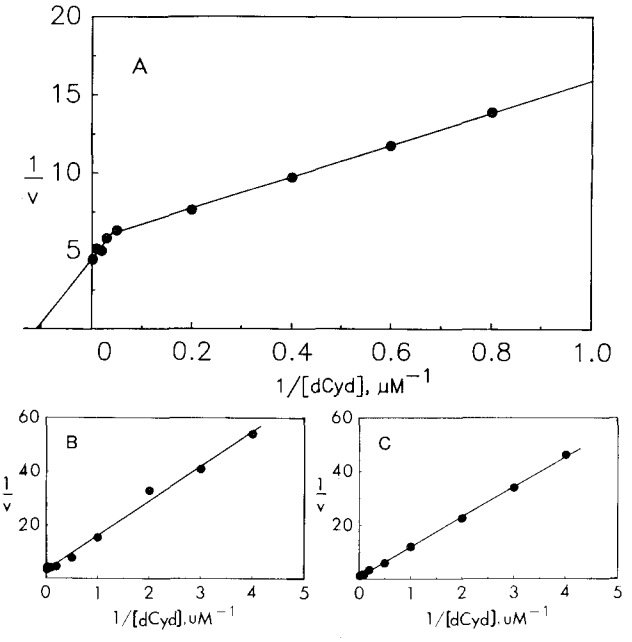

Figure 3. Lineweaver-Burk kinetics of mixture of parental and proteolyzed dCyd kinase. Each 80 ul reaction mixture contained 0.19 ug parental enzyme and/or 0.16 ug proteolyzed enzyme. ATP was 10 mM and $MgCl_2$ was 12 mM.

A. Mixture of parental and proteolyzed enzyme proteins. (The two slopes were individually fitted by computer.)
B. Parental protein fraction only.
C. Proteolyzed fraction only.

qualitative rather than quantitative terms. The results of comparative isoelectric focusing were less dramatic, in that the principal pI appears to have shifted only slightly in the proteolyzed sample, from pH 6.1 to 6.2 (results not shown). Within the range of experimental error, this pI is unchanged. However, a minor peak was detected at pH 6.9-7.0.

Kinetic effects of proteolysis--We wondered whether a mixture of the parental and proteolyzed enzyme might account for the non-linear kinetics often observed with dCyd kinase. Although other human leukemic cell extracts studied in this laboratory have yielded such broken line kinetics (unpublished work), with this ALL cell preparation we did not succeed in trapping the optimum blend of molecular species which would show this most clearly. Therefore, we mixed approximately equal amounts of proteolyzed and unproteolyzed protein, with the kinetic results shown in Figure 3. Apparent K_m values of 1.8 and 9.3 uM were obtained for dCyd from the slopes of this plot, 7A. Individually (7B & C), each preparation yielded a straight line, but the proteolyzed enzyme appeared to have a significantly larger K_m^{app} (13 uM, compared with 3.3 uM for the parental enzyme).

DISCUSSION

We have described a simple and efficient two-step method for isolating apparently homogeneous deoxycytidine kinase protein by affinity chromatography. It is believed to be the first time an affinity ligand of this degree specificity has been applied to the purification of dCyd kinase from mammalian cytosol. Ligands based on structural analogs of nucleoside triphosphates, including Blue Sepharose, have been useful but not completely selective (8, 10, 25). On the other hand, most modifications of the substrate towards which the enzyme is most specific--the deoxynucleoside--drastically interfere with binding (26).

The enzyme has a small apparent K_m for dCyd, but exhibits much weaker affinities for dAdo and dGuo. If the crude leukemia cell extract should also contain low-K_m versions of dAdo and dGuo kinase activities, as have been reported in T-lymphoblasts (6), they were not recovered from the dCp_4A-Sepharose column. Perhaps the dAp_4A-Sepharose bisubstrate medium (14) would be more specific for this putative isoenzyme.

The discovery that proteolysis can occur on the affinity column--when the enzyme protein is very dilute--highlights the very important concept that, unless suitable precautions are taken, an enzyme isolated from human leukocytes may be a modified protein. Fortunately, a mixture of simple chemical inhibitors appeared to completely eliminate this effect. A similar proteolytic phenomenon was reported for calf thymus terminal deoxynucleotidyl transferase (27, 28). Crude thymus extracts were found to contain 58 and 44 kDa active enzyme peptides, in addition to the 32 kDa peptide species originally isolated as terminal transferase, and it was suggested that the alpha and beta subunits reported in the 32 kDa preparation may have arisen by proteolysis of the 58 kDa monomeric peptide (28). Evidence has also been cited recently that human DNA polymerase δ is modified during isolation from placental extracts (29). By analogy, it seems quite possible that the T-cell specific kinase reported as having a molecular weight of 26.5 kDa, (30), may have been a proteolyzed fraction, with corresponding changes in its kinetic properties, as well.

The proteolysis we have observed appears to have been a discrete cleavage producing two fragments of unequal size. SDS-gel electrophoresis revealed a parental polypeptide unit of about 52 kDa, which is comparable to the sum of the fragments and to the molecular weight of the native protein. These results are in agreement with the other estimates of the M_r of human dCyd kinase, which range from 53-56 kDa (9, 10, 30) to 68 kDa (31).

The kinetic pattern we obtained with a synthetic mixture of parental and proteolyzed proteins bears a striking resemblance to results obtained with dCyd kinase isolated without protease inhibitors from other human T-lymphocytic cells (2, 8), and from calf thymus dCyd kinase (12, 13). There seems to be a distinct possibility, therefore, that limited proteolysis of the kinase may account for the non-linear kinetics obtained in several cases. Further, if the modified protein were the form of the enzyme isolated, the K_m values and other physical properties reported might be subject to error. While we can minimize this possibility by the judicious use of protease inhibitors, we will not be certain that even the 52 kDa polypeptide is unmodified until its terminal sequences are compared with the corresponding genetic sequences.

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