Characterization and purification of a novel dATP-binding protein in eukaryotes

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We characterized and purified an acidic dATP-binding protein, which, in its active form, resides in the nuclear fraction of a range of cells from mammals (including pig liver) and baker's yeast (*Saccharomyces cerevisiae*). This protein exhibits a high degree of specificity for the deoxy form of the naturally occurring nucleoside triphosphates and shows a marked preference for the purine deoxynucleoside triphosphates dATP and dGTP. The protein cleaves the terminal phosphate of dATP and appears to retain the dADP moiety of the nucleotide in a reaction that is resistant to both SDS and 8 M-urea. Fractionation of the nuclear preparation followed by non-denaturing PAGE and SDS/PAGE electrophoresis was sufficient to produce pure protein. The occurrence of this activity in all nuclei tested suggests that it plays an important role in nuclear metabolism. The specificity of the enzyme for deoxynucleoside triphosphates further suggests a role for this enzyme in DNA replication or repair, but the acidity of the protein argues against a direct interaction with DNA, and, indeed, the catalytic activity is not modulated by the inclusion of DNA in a variety of physical forms.

INTRODUCTION

During the course of an investigation into nuclear proteins and their interaction with nucleotides we discovered a deoxy-nucleotide-specific binding protein present in a wide range of eukaryotes from yeast to humans. Here we describe the characterization of the physical properties of the protein, its substrate specificity, a preliminary investigation into the kinetics of the ligand binding and its enzyme activity, together with its purification. An extensive review of the literature failed to reveal a record of any similar activity, and we thus conclude that the activity is completely novel. The high degree of functional conservation throughout the eukaryotes and the characteristics herewith described are suggestive of a role in the regulation of processes such as nuclear signalling, DNA replication repair or recombination.

MATERIALS AND METHODS

Preparation of nuclear extracts

Nuclei were obtained from rat and pig liver by homogenizing fresh livers in a sucrose buffer (final concns: 1.6 M-sucrose, 80 mM-NaCl, 1 mM-EDTA, 20 mM-Hepes, pH 7.9, 2 mM-dithiothreitol, 0.5 mM-spermidine and 0.15 mM-spermine), followed by centrifugation on a sucrose cushion (buffer as just described, but with the addition of 1.8 M-sucrose) as described [1]. The nuclear pellet was homogenized [2], the nuclear protein fraction was separated by centrifugation and then dialysed against the glycerol/Hepes buffer described in [3]. For preparative electrophoresis, nuclei were resuspended in a buffer containing 20 mM-Hepes, pH 7.9, 2% (v/v) glycerol, 0.42 M-NaCl, 1.5 mM-MgCl₂, 0.2 mM-EDTA and 0.5 mM-phenylmethanesulphonyl fluoride and, after stirring for 45 min, the ruptured nuclei were centrifuged at 27 000 g for 20 min. The dATP-binding activity was recovered from the pellet by a brief sonication in a buffer containing 20 mM-Hepes, pH 7.9, 2% (v/v) glycerol, 20 mM-KCl, 2 mM-MgCl₂, 0.2 mM-EDTA and 0.5 mM-dithiothreitol. Extracts pre-

pared in this way were then dialysed against the final buffer containing 20% (v/v) glycerol and stored at -70 °C.

Whole-cell extracts were prepared from baker's yeast (*Saccharomyces cerevisiae*). The growth and preparation of cell-free extract was essentially as described in [4], but with the solubilization buffer as described previously, i.e. 20 mM-Hepes, pH 7.9, 20% glycerol, 0.42 M-NaCl, 1.5 mM-MgCl₂, 0.2 mM-EDTA and 0.5 mM-phenylmethanesulphonyl fluoride [2], with the addition of proteinase inhibitors leupeptin (final concn. 0.5 µg·ml⁻¹), aprotinin (1.0 µg·ml⁻¹) and pepstatin (0.7 µg·ml⁻¹). The cellular proteins were then dialysed as in the mammalian preparations.

Assays

The standard assay mixture for monitoring dATP binding by the protein extracts contained the following: 2 µl (10 µg) of protein extract, 0.06 pmol of [α -³²P]dATP in a buffer comprising 75 mM-Hepes, pH 7.9, 65% (v/v) glycerol, 180 mM-NaCl, 2 mM-dithiothreitol and 2 mM-phenylmethanesulphonyl fluoride [5] in a total volume of 20 µl. All incubations were performed at 30 °C for 20 min. Variations in this standard incubation are described in the Figure legends.

In order to detect dATP-protein complexes incubation mixtures were run on acrylamide/bisacrylamide (3%:0.1%) non-denaturing gels at 12.5 V·cm⁻¹ for 1.74 h. The gels were dried and exposed to film as required.

Determination of binding affinities

A 2 µl (10 µg) portion of nuclear protein was incubated with [α -³²P]dATP concentrations (three replicates per concentration) covering the range 0.0556 nM to 5.56 nM and electrophoresed on four separate non-denaturing gels simultaneously, Band B (see Fig. 2 below) was cut out together with a background control section of gel of similar size for each lane. The gel sections were thoroughly dried under vacuum then dissolved in H₂O₂ and a normalized estimate of c.p.m. (n.c.p.m.) obtained by scintillation

Abbreviations used: dAMPCP, 5'-O-deoxyadenosyl P1 ester of bis(dihydroxyphosphinomethyl)phosphinic acid; ddATP, dideoxyATP; bdATP, biotinylated dATP.

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counting as described elsewhere [6]. Control samples (three replicates) at each ligand concentration were counted simultaneously. The background value of n.c.p.m. for each lane was substrated from the value for each band, and this derived value, in conjunction with internal standards of free dATP, was used to calculate the respective concentrations of free and bound nucleotide.

Chromatography and electrophoresis

Polygram Cel 300 DEAE t.l.c. was carried out according to the manufacturers instructions using 0.075 M-potassium phosphate, pH 7.5, as a developing buffer.

H.p.l.c. gel filtration was carried out with a TSK-250 column (Bio-Rad) in 50 mM-potassium phosphate/150 mM-NaCl buffer, pH 7.5. Samples (1 ml each) were collected into 0.3 ml of glycerol containing dithiothreitol (0.5 mM final concn.). The assays were standard, except that 19 μ l of each sample were used, since buffering was already approximately correct for the binding reaction.

F.p.l.c. ion-exchange chromatography was carried out using a MonoQ anion-exchange column in the glycerol/Hepes buffer over a gradient of 0.02 M–2 M-KCl. Samples (1 ml) were collected and the assays were standard, except that 18 μ l of each sample was used with the addition of NaCl (0.036 M final concn.) to each incubation to correct buffering.

SDS/PAGE was performed in 1 $\times$ TBE buffer (89 mM-Tris/borate/89 mM-boric acid/2 mM-EDTA, pH 8.3) in a continuous system.

Preparation of 5'-O-deoxyadenosyl P1 ester of bis(dihydroxyphosphinomethyl)phosphinic acid (dAMPCPCP)

The non-hydrolysable analogue of dATP, dAMPCPCP, was synthesized by applying the methodology of Poulter [7] for the displacement of a tosyl group by a bisphosphonate anion.

The 5'-O-tosyl 2'-O-deoxyadenosine was prepared by adapting the synthesis of 5'-O-tosylthymidine [8]. The product was purified by column chromatography on silica gel in 62% yield.

Bis(dihydroxyphosphinomethyl)phosphinic acid was prepared by methods previously described [9–11].

While this work was in progress the ribose derivative AMP-CPCP was prepared by a method similar to that used in [12].

Bis(dihydroxyphosphinomethyl)phosphinic acid (190 mg, 2.2 mmol) at a concentration of 33 M in water was titrated to pH 7.7 with 40% (w/v) tetrabutylammonium hydroxide to produce the tetrakis(tetrabutylammonium) salt. Solvent was subsequently removed *in vacuo* and the salt dried by repeated evaporation of anhydrous pyridine (3 vol.) and dry acetonitrile (3 vol.) and maintained *in vacuo* for 12 h. The salt was dissolved in acetonitrile (2.5 ml), and 5'-O-tosyl 2'-deoxyadenosine (200 mg, 0.5 mmol) was added and the reaction mixture stirred

for 72 h. Solvent was removed by evaporation and the product purified by three consecutive passes down a column of DEAE Sephadex A-25 resin (Pharmacia) developed with 6 litres of a 0–0.34 M gradient of triethylammonium bicarbonate. The purified product, which was eluted at a salt concentration of 210 mM, was converted into the sodium salt by dissolution in a minimum volume of methanol and precipitated with 1 M-NaI in acetone and collected by centrifugation after several acetone washes. From 190 mg of starting material, 34 mg of pure product (12% yield) was obtained, calculated from 5'-O-tosyl-2'-O-deoxyadenosine. The purity and composition of the product (Fig. 13 below) was determined by f.p.l.c. on a Pharmacia Mono Q column eluted with a linear gradient of 0–1 M-NH₄HCO₃, mass spectroscopy (Kratos MS 80RF model with a DS90 data system), ¹H n.m.r. (Bruker AM 250 at 250 MHz.) and ³¹P n.m.r. (Bruker WP 80 SY at 32.4 MHz). The data are given below.

$\delta^{31}\text{P}(\text{H}_2\text{O})$ 29.4 (1P dd, $^2J_{\text{pp}}$ 12.4 Hz, $^3J_{\text{pp}}$ 8.6 Hz, P²), 19.3 (1P, d, $^2J_{\text{pp}}$ 12.4 Hz, P¹), 13.4 (1P, d, $^2J_{\text{pp}}$ 8.6 Hz, P³); $\delta^1\text{H}$ 8.42 (1H, s, H⁸), 8.13 (1H, s, H²), 6.41 (1H, dd, $J_{6,5}$ 5 Hz, H¹), 4.48–4.72 (1H, m, H³), 4.15–4.22 (1H, m, H⁴), 3.92–4.10 (2H, m, H⁵, H^{5'}), 2.76 (1H, ddd, $J_{\text{H}^2\text{H}^3}$ 14.0 Hz, $J_{\text{H}^2\text{H}^1}$ 6.5 Hz, $J_{\text{H}^2\text{H}^4}$ 1.0 Hz, H²), 2.50 (1H, ddd, $J_{\text{H}^3\text{H}^2}$ 14.0 Hz, $J_{\text{H}^3\text{H}^1}$ 6.5 Hz, $J_{\text{H}^3\text{H}^4}$ 4.0 Hz, H³), 2.25 (2H, dd, $^2J_{\text{HP}}$ 21.0 Hz, $^3J_{\text{HP}}$ 16.5 Hz, PCH₂P), 2.01 (2H, ddd, $^2J_{\text{HP}}$ 18.5 Hz, $^4J_{\text{HP}}$ 1.0 Hz, PCH₂P); m/z (fast-atom bombardment + ve), 576 (100%, $M + 4\text{H}^+ - 3\text{H}^+$), 598 (42%, $M + 5\text{Na}^+ - 4\text{H}^+$), 554 (50%, $M + 3\text{Na}^+ - 2\text{H}^+$) in the range 400–650. C₁₂H₂₀O₁₀N₅P₃ Mr 487; h.p.l.c. R_t (retention time) 6.0 min. Chemical shifts δ_{H} are quoted in p.p.m. downfield of the external capillary. Chemical shifts δ_{P} are quoted in p.p.m. downfield of the 85% (w/v) orthophosphoric acid external reference.

Miscellaneous

All radiolabelled nucleotides were obtained from Amersham International, non-radioactive nucleotides were obtained from Boehringer, and enzymes were purchased from Sigma.

RESULTS

DeoxyATP binding activity in crude nuclear extracts

A series of gel-retardation experiments on nuclear extracts from rat and pig liver revealed the presence of a common factor or factors which bound [α -³²P]dATP (e.g. Fig. 1a). This activity was reproducibly detected using a modification of the nuclear extraction procedure described in [2] and by the inclusion of glycerol at a concentration of 20% at all stages of the preparation (see the Materials and methods section). A major fast-migrating species (designated B in Fig. 1a) was consistently found in extracts prepared from both cell sources (and from many other cell types, including HeLa cells). In addition, a second, slower-migrating, species, A (see Fig. 1a), was always detected in pig liver nuclear extracts but, from subsequent experiments, appears to be present in a more labile form in rat liver preparations. Yeast whole-cell extract prepared in 20% (v/v) glycerol contained similar dATP-binding activities. However, whereas the major fast-migrating species, B, appeared to be identical with that of the mammalian preparations, the slower-migrating species, A, showed higher mobility when electrophoresed and may not be related to the corresponding species in mammalian preparations.

In order to determine the nature of the molecule(s) involved in this binding interaction, the crude extract was treated separately with DNAase I, RNAase A and proteinase K before the addition of [$\delta^{32}\text{P}$]dATP and subsequent electrophoresis. The factor which forms a complex with dATP is clearly identified as a protein, since only treatment of the extract with proteinase K led to

Fig. 1. Nucleotide specificity of the nuclear extracts

(a) dATP binding activity and specificity in rat and pig liver nuclear extract and yeast whole-cell extract. Lanes 1-3, standards (1, rat nuclear extract; 2, pig nuclear extract; 3, yeast whole-cell extract). Lane 4, as 3, but with 4 µl (20 µg) of yeast whole-cell extract. (b) Lane 1, standard assay conditions; 2-6, as standard, but with the following deoxynucleotides and ribonucleotides replacing the $[\alpha\text{-}^{32}\text{P}]\text{dATP}$; 2, $[\alpha\text{-}^{32}\text{P}]\text{ATP}$; 3, $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$; 4, $[\alpha\text{-}^{32}\text{P}]\text{dGTP}$; 5, $[\alpha\text{-}^{32}\text{P}]\text{dTTP}$; 6, $[\alpha\text{-}^{32}\text{P}]\text{UTP}$. The radioactivity of each nucleotide was approximately equal.

abolition of the retarded band. Moreover, the binding of the ligand was not stimulated by the presence of either endogenous RNA or DNA or by the addition of supercoiled, relaxed or linearized plasmid DNA under the conditions of the assay.

Nucleotide specificity

A number of proteins have been described, including numerous kinase enzymes that are able to substitute dATP for the more usual ATP substrate with varying degrees of efficiency [13]. On the other hand, enzymes associated with DNA biosynthesis, such as DNA polymerases and ribonucleotide reductase, exhibit a high degree of specificity for the deoxy forms of ATP, GTP, TTP and CTP. We set out, therefore, to establish whether this activity was indeed different from those described above by using gel-retardation assays.

In Fig. 1(b) the results of gel-retardation experiments using $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (Fig. 1b, lane 2) or $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ (Fig. 1b, lane 6), clearly demonstrate that dATP is not substituting for ATP in the binding reaction. In contrast, all of those $\alpha\text{-}^{32}\text{P}$ -labelled deoxynucleotides examined showed binding (Fig. 1b, lanes 1, 3-5), but the extent of retardation differed slightly with dCTP and dTTP compared with dATP and dGTP. The protein also appeared to have a lower affinity for the pyrimidine deoxynucleotides, since the bands produced were less intense (the specific activities and the amounts of ligands used being essentially the same in each experiment). Subsequently, these results were confirmed by competitive binding studies using the partially purified preparation; a 10-fold excess of unlabelled pyrimidine deoxy-

Fig. 2. Differential labelling with $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ and $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ with time

Lanes 1, 3, 5, 7 and 9 are standards with incubation times of 0, 5, 10, 20 and 40 min respectively; Lanes 2, 4, 6, 8 and 10 are as the standards, but with $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ as the ligand. The incubation times are as given above. The radioactivity of the $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ was approximately double that of $[\alpha\text{-}^{32}\text{P}]\text{dATP}$.

nucleotide compared with unlabelled dATP was required to achieve a similar reduction in binding of labelled dATP (800 pmol of unlabelled dCTP or dTTP and 80 pmol of unlabelled dATP produced a comparable reduction in binding of 0.06 pmol of $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ added subsequently).

Characterization of the bound ligand

To establish whether hydrolysis of any portion of the ligand occurred upon binding to the protein $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ and $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ were employed as substrates in comparative gel-retardation assays. The results of an experiment in which the radiolabelled substrates were added to a crude nuclear extract for a series of timed incubations are shown in Fig. 2. Clearly, the fast-migrating species does not contain intact dATP, but must contain a portion of the molecule, such as dADP, dAMP or at least the α -phosphate. Moreover, the results shown in Fig. 2 give a preliminary indication of the kinetics of the binding reaction. Under the conditions of the experiment it appears that at least the terminal phosphate is hydrolysed from dATP before formation of the stable protein complex, since within the time scale of the experiment, no complex with the γ -phosphate species is seen in the position normally occupied by band B. It is not possible to draw any conclusions at this stage about the significance of the increase in intensity of the upper band (A) in Fig. 2, since this band is often observed when either $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ or $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ are used as ligands (see Fig. 1a, lane 2). Band A has also been seen with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Fig. 4 below, lanes 11 and 12), strongly suggesting that this is not a deoxynucleotide-specific reaction. It is also clear from Fig. 2 that the specific dATP binding reaction reaches a maximum after between 10 and 20 min incubation under the standard assay conditions.

Analysis of the dATPase reaction

In order to establish whether this protein liberates phosphate or pyrophosphate on binding dATP, we used t.l.c. to monitor the appearance of the products of the incubation (Fig. 3). Phosphate release was detected with $[\gamma\text{-}^{32}\text{P}]\text{dATP}$, but also with the more conventional phosphate donor $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (perhaps not unexpected in a crude nuclear extract). There was no evidence of the release of pyrophosphate with either ligand. However, it is possible that, with dATP as ligand, the initial hydrolysis occurred between the α - and β -phosphates, and that further hydrolysis of the PP_i to P_i phosphate occurred as a secondary reaction in the nuclear extract.

Fig. 3. Demonstration of P_i release in nuclear extracts using $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ and t.l.c.

All samples were incubated for 20 min at 30 °C in a total volume of 20 μl of buffer, thereby removing any effect of buffer or incubation; 1 μl of each sample was loaded. Lane 1, ^{32}P ; 2, ^{32}P and nuclear extract; 3, $[\gamma\text{-}^{32}\text{P}]\text{dATP}$; 4, $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ and nuclear extract; 5, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$; 6, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and nuclear extract.

Fig. 4. Gel filtration of pig nuclear extract, showing differential binding activity of $[\alpha\text{-}^{32}\text{P}]\text{dATP}$, $[\gamma\text{-}^{32}\text{P}]\text{dATP}$ and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.

A 25 μl (125 μg) portion of pig nuclear extract was eluted through a TSK-250 gel-filtration column as described in the Materials and methods section. Binding activity was recovered in fractions 2-4 and fraction 7+8 (heavily stippled area and inset) using $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ and fractions 9 and 10 (lightly stippled area and inset) using $[\gamma\text{-}^{32}\text{P}]\text{dATP}$. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ produced similar binding to $[\gamma\text{-}^{32}\text{P}]\text{dATP}$. Incubations were as described in the Materials and methods section. Lanes 1-4, fractions 7-10 labelled with $[\alpha\text{-}^{32}\text{P}]\text{dATP}$; 5-8, fractions 7-10 labelled with $[\gamma\text{-}^{32}\text{P}]\text{dATP}$; 9-12, fractions 7-10 labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$.

Fractionation of the dATP binding activity

In view of the uncertainties inevitably associated with experiments in crude extracts, we conducted a series of preliminary fractionation experiments using h.p.l.c. gel filtration and anion-exchange chromatography. The elution profile obtained after chromatography of pig liver nuclear extract on a Bio-Rad TSK-250 gel-filtration column is shown in Fig. 4. Simultaneous gel-retardation assays on the eluted fractions revealed two things. First, that the activity was eluted at a position consistent with the protein having a molecular mass of 200 kDa (although a very

Fig. 5. Mono Q ion-exchange gradient elution of rat nuclear extract dATP-binding activity

(a) A 100 μl (500 μg) portion of rat nuclear extract was eluted with a 0.02-2 M-KCl gradient in buffer [5]. dATP-binding activity was recovered in fractions 28-36 (stippled area and b) using the standard gel-shift assay and 16 μl of each 1 ml fraction.

small amount of activity was eluted in the void volume) and, secondly, the slow migrating band (A) associated with both ATP and dATP is clearly distinct from the dATP specific binding activity. Indeed, the non-specific ATP activity is associated with a protein (or proteins) of molecular mass 80 kDa. SDS/PAGE of the active fraction followed by Coomassie Blue staining showed a large number of protein species present.

When the nuclear extract was fractionated by f.p.l.c. using a Mono Q anion-exchange resin (Pharmacia) in glycerol/Hepes buffer, pH 7.9 [3], the dATP binding fraction was eluted at a concentration of 2 M-KCl (Fig. 5), which suggests that the protein is acidic. This observation is consistent with the relatively fast migration of the complex on non-denaturing polyacrylamide gels (compare band B with band A in Fig. 1a). SDS/PAGE of the pooled active fractions followed by silver staining showed no

Fig. 6. Silver-stained non-denaturing gel of unbound protein

Increasing amounts of pig nuclear extract were electrophoresed under standard non-denaturing conditions and silver-stained. Lane 1, 5 μ l (5 μ g); 2, 10 μ l (10 μ g); 3, 20 μ l (20 μ g).

Fig. 7. dATP-binding activity eluted from an excised band

Lane 1, standard (pig); 2, 2 $\times$ standard to control for loading volume; 3, 5 μ l of eluate in standard incubation mixture (total volume 20 μ l); 4, 10 μ l of eluate in standard incubation; 5, 20 μ l of eluate in standard incubation ($\times$ 2). Lanes 3–5 were exposed to autoradiographic film approx. 3-fold longer.

major protein species, and the dATP-binding activity of the 2 M-salt fractions from the Mono Q was unstable.

Since conventional chromatography proved of limited use in purifying the dATP-binding activity, we exploited the acidity of the protein in a preparative-electrophoresis procedure. As Fig. 1 shows, the active protein species migrates to the foot of the gel, consistent with its elution at high salt from Mono Q. When nuclear extracts were prepared in a high-viscosity buffer (see the Materials and methods section), it was difficult to exclude chromatin, fragmented nuclear envelope and rough endoplasmic reticulum from the preparation. By reducing the glycerol concentration from 20% (v/v) to 2% (v/v), after rupture of the nuclei, it was possible to resolve the original active fraction into an insoluble and a soluble phase by centrifugation. The bulk of

the dATP-binding activity resided in the insoluble fraction, but could be released by sonication. This sonicated extract was subsequently loaded on to a non-denaturing gel in the presence or absence of radioactively labelled dATP. The band which migrated furthest (see Fig. 6) coincided with the dATP-binding activity. Moreover, the protein eluted from this excised band gave dATP binding activity (Fig. 7), and gel electrophoresis of the eluted protein in the presence of SDS gave two bands of molecular masses 60 kDa and 24 kDa (Fig. 8). It is clear that these two bands are not part of a single complex, since by physically dividing the silver-stained gel slice, two polypeptides could be resolved on SDS/PAGE of subunit molecular mass 60 kDa and 24 kDa. The 24 kDa band is by far the major species. Although gel filtration provided a stable active fraction, there was very little purification achieved, and in view of the instability of the Mono Q fractions, the preparative-electrophoresis step proved more reliable. However, although this method proved satisfactory for obtaining a near-homogeneous preparation of the pig liver dATPase, it proved unsuitable on a preparative scale, both in yield and stability of the protein. However, we were able to confirm that the 24 kDa polypeptide (or, less likely, the 60 kDa species) was responsible for dATP binding. Thus we subsequently used the partially purified fraction (Fig. 6) for further characterization of the protein. This fraction showed the same ligand specificity as the crude extract, but we were unable to characterize the bound ligand as described for Figs. 2 and 3 as [γ - 32 P]dATP was no longer available. Thus we focused our subsequent investigations on further characterization of the ligand specificity and the binding kinetics, as neither of these factors appeared to be affected by impurities in the preparation, and we had established from the results in Fig. 7 that a band containing only two protein species could bind dATP in a manner similar to the partially pure preparation.

Binding affinity studies

Competition experiments using the partially pure preparation were carried out with dideoxyATP (ddATP) and biotinylated dATP (bdATP). When ddATP was incubated with the nuclear extract in advance of labelled dATP, there was no effect at all on complex formation, even at over 10^4 -fold excess of the dideoxy analogue (Fig. 9a). It is clear that the ligand binding site of the enzyme–enzyme complex is highly specific for the 2'-deoxy form of the ribonucleotide; the presence of an additional oxygen on the ribonucleotide, as in ATP, or the absence of both (in ddATP), prevent binding. Competitive binding assays using bdATP (Fig. 9b) showed this compound to be only 20-fold less effective as a ligand than dATP (800 pmol of biotinylated dATP and 40 pmol of unlabelled dATP produce a comparable reduction in binding of 0.06 pmol of [α - 32 P]dATP added subsequently). Therefore whereas the presence of biotin at the 6-position of the purine ring interferes significantly with the formation of the enzyme–substrate complex, it is more easily accommodated than the other substitutions investigated. It is conceivable that bdATP may prove useful as a ligand in the synthesis of an affinity column for the purification of this protein.

It was clear from these experiments that the affinity of the enzyme for its ligand was very high (1000-fold excess of unlabelled dATP could not totally prevent subsequent binding of [α - 32 P]dATP (Fig. 9b, lanes 6–9). However, the quantification of a gel-retardation experiment in which the protein was titrated against [α - 32 P]dATP showed that no end point is reached under the standard assay conditions. This suggests either that this protein binds dATP in both a specific and a non-specific manner or, perhaps more likely, that at least two complexes between ligand and protein exist. For example, it is possible that dATP initially forms a reversible complex with free protein before the

Fig. 8. TBE/SDS/PAGE of excised band

The arrows indicate the two protein species eluted

Fig. 9. Competitive binding of nucleotide analogues in pig nuclear extract

(a) Lane 1, standard (2 μ l of extracts, 2 μ g of protein); 2–4, 2 μ l of extract incubated with decreasing concentrations of ddATP (480, 80 and 8 pmol respectively), followed by the addition of 0.06 pmol [α - 32 P]dATP as a secondary incubation; 5–7, 2 μ l of extract incubated with decreasing concentrations of dATP (as in 2–4), followed by the addition of 0.06 pmol of [α - 32 P]dATP as a secondary incubation. (b) Lane 1, standard; 2–5, 2 μ l of extract incubated with decreasing concentrations of bdATP (480, 80, 8 and 4 pmol respectively), followed by the addition of 0.06 pmol of [α - 32 P]dATP as a secondary incubation; 6–9, 2 μ l of extract incubated with decreasing concentrations of dATP (as in 2–5), followed by the addition of 0.06 pmol of [α - 32 P]dATP as a secondary incubation.

formation of a covalent (or at least a high-affinity non-covalent) complex.

In order to explore the above hypothesis, we looked for evidence for the presence of an irreversible complex between the protein and dATP using competition assays with unlabelled dATP and also by denaturing PAGE and by treatment of the complex with 8 M-urea.

If a single reversible complex is formed between the protein and dATP, then incubation of the extract with unlabelled dATP, either before or after the addition of radioactive dATP, should yield the same result. In Fig. 10 it is quite apparent that the addition of labelled dATP to the nuclear extract leads to the formation of a very tight protein–ligand complex which cannot be significantly dissociated even by the addition of an over- 10^8 -fold excess of unlabelled ligand (Fig. 10, lanes 2–6). In contrast, the complex formed after preincubation of the extract with unlabelled dATP can undergo a detectable exchange with radioactive dATP when the unlabelled dATP is added at nanomolar concentrations and is challenged with 10^5 -fold lower con-

Fig. 10. Competitive binding with dATP

Lane 1, standard (rat nuclear extract); 2–6, standard incubations followed by secondary incubation with increasing amounts of unlabelled dATP (10 nmoI, 100 nmoI, 1 μ mol, 2 μ mol and 4 μ mol); 7–11, 2 μ l of extract incubated with decreasing concentrations of unlabelled dATP (4 μ mol, 2 μ mol, 1 μ mol, 100 nmoI and 10 nmoI), followed by the addition of 0.06 pmol of [α - 32 P]dATP.

Fig. 11. SDS/PAGE of protein–ligand complex

Lane 1, standard (rat nuclear extract) loaded in non-denaturing-gel loading buffer; 2, as 1, except with pig nuclear extract; 3, standard (rat) boiled and loaded under reducing conditions; 4, as 3, except using pig nuclear extract; 5, a combination of four standard incubations of rat nuclear extract dialysed against 4 litres of 0.1% SDS and then boiled and loaded under reducing conditions; 6, as 5, except using pig nuclear extract. Labelled protein species are indicated by arrows.

centrations of the labelled ligand (Fig. 10, lane 11). These results support the hypothesis that, after the addition of dATP to the protein, there is a transient formation of a reversible complex

Fig. 12. Competitive binding with a non-hydrolysable analogue of dATP, dAMPCPCP, in pig nuclear extract

Lane 1, standard; 2–5, 2 μ l of extract incubated with decreasing concentrations of non-hydrolysable analogue (480, 80, 8 or 4 pmol respectively), followed by the addition of 0.06 pmol of [γ - 32 P]dATP as secondary incubation; 6–9, 2 μ l of extract incubated with decreasing concentrations of dATP (as in 2–5), followed by the addition of 0.06 pmol of [α - 32 P]dATP as a secondary incubation.

followed by the formation of a second, possibly irreversible, complex.

To investigate the possibility of the formation of a covalent enzyme–dATP complex, we dialysed a standard incubation mixture against 8 M-urea. There was less ligand bound when compared with an untreated control incubation, but still clearly some dATP had remained bound after the denaturing treatment (results not shown). In order to determine whether a stable ligand–protein complex could be isolated, we electrophoresed a standard incubation mixture on an SDS/polyacrylamide gel. The result shown in Fig. 11 clearly shows the presence of a range of radiolabelled protein species. These were obtained after extensive dialysis of the pig liver nuclear extract (after incubation with labelled dATP) against 1% SDS and subsequent boiling of the extract in the presence of β -mercaptoethanol; at this stage we are unable to interpret the SDS/PAGE data with any certainty, owing to the large number of labelled species obtained; they may be proteolytic degradation products.

Competition assays with dAMP and dADP using both crude and partially pure preparations provided further support that the retained moiety was dADP as suggested from the results of the assays with [γ - 32 P]dATP. dADP as a ligand was as efficient a competitor for the protein as dATP, whereas dAMP showed an approx. 10-fold weaker effect in competitive binding assays. Therefore, although it is likely that this enzyme hydrolyses dATP to dADP with the retention of the dADP moiety, absolute confirmation of this interpretation must await purification of a sufficient quantity of the enzyme. It also suggests that, if two separate proteins are involved in the binding and hydrolysis of the ligand, these proteins co-purify upon gel filtration and anion-exchange chromatography and both are associated with the solid phase of the nuclear extract. Purification of a sufficient quantity of stable protein should enable one to establish, using [γ -

32 P]dATP, whether it is a single protein species responsible for both binding and catalysis. In view of the fact that the bound ligand is not dATP, but a portion of it, and the possibility of two enzyme–substrate complexes, as suggested by the binding-affinity studies, we propose that the first transient complex is formed between a protein and dATP, whereas the second, possibly covalent, complex is between a protein and dADP.

In order to dissect the two possible binding events, we synthesized a non-hydrolysable dATP analogue, dAMPCPCP (see the Materials and methods section). The results, shown in Fig. 12, show that this analogue mimics dATP in that equivalent amounts of dATP and dAMPCPCP will prevent subsequent binding of radiolabelled dATP. This reagent should therefore prove invaluable in characterizing the two binding events in the mechanism of action of this enzyme.

DISCUSSION

In a simple, but revealing, gel-retardation assay, we have identified a functionally conserved nuclear dATPase protein in a range of nuclei which appears to bind the nucleotide diphosphate product of the hydrolysis reaction in a complex, but specific, manner. The two activities are co-eluted after both gel-filtration and anion-exchange chromatography. Moreover, after nuclear extraction and electrophoresis under non-denaturing conditions, excision of an acidic band which co-migrates with the dATP binding activity and subsequent electrophoresis under dissociating conditions results in the appearance of two protein species of molecular masses 24 and 60 kDa. The former is the major component. It appears, therefore, that the dATP binding and hydrolysis is catalysed by a multimeric complex of the 24 kDa polypeptide and that this enzyme binds all four naturally occurring deoxynucleotide triphosphates (dNTPs) with differing affinities (and different electrophoretic properties; see Fig. 1b), and we suggest that dCTP, dGTP and dTTP would be similarly hydrolysed. We have chosen to characterize and purify the dATP-binding activity in nuclear extracts as a first stage in the investigation of this activity.

We have established that the protein or protein complex involved in this activity has stringent requirements for ligand binding. It seems that only the 2'-deoxy form of the nucleotide is able to bind in a catalytically competent manner. The presence of a 2'-OH group or the removal of both the 2'-OH and the 3'-OH abolishes binding (see Fig. 13).

In order to explore ligand binding by this enzyme we have synthesized a novel non-hydrolysable analogue of dATP which, at approximately the same concentrations, can mimic the effect of dATP in competing out the reversible dATP binding activity associated with this protein (see Fig. 12). Thus the presence of methylene groups in place of the phosphodiester linkages, although sufficient to render the ligand ineffective as an inhibitor of the dATPase reaction, does not prevent initial complex-formation. This ligand will need to be prepared in a radioactive form if one is to resolve the complex nature of the binding reaction observed in Fig. 12. In addition, the substitution of an N⁶ proton on the adenine by biotin is seemingly permissible (see Fig. 9b). As Fig. 13 shows, the most drastic reduction in ligand binding is observed when the deoxy form of the ribose sugar is replaced with either the ribose or the dideoxy form. However substitutions in the adenine moiety are tolerated more readily. This pattern of substrate selectivity is reminiscent of that found in DNA polymerase enzymes, in which discrimination between ribose and deoxyribose is an essential component of the enzyme's biological function. However, whereas ddNTPs are often potent inhibitors of DNA polymerases, the presence of a 3'-OH group in the case of the dNTPase is absolutely essential for ligand

Fig. 13. Representation of relative binding affinities of dATP analogues

binding. Indeed ddATP is unable to displace dATP even at a molar excess of 10^5 . We know of no other examples of enzymes or proteins which show such selectivity between these nucleotides. The implication of these results is that a single hydrogen bond between the 3'-OH of the sugar moiety and the enzyme is absolutely essential for ligand binding. The molecular interactions between this system and the dNTPs presents a fascinating problem in molecular recognition (Fig. 13).

The dATP binding activity of the enzyme is independent of both DNA and RNA, and ion-exchange fractionation of the enzyme (Fig. 5) shows clearly that the protein is acidic. This latter property is perhaps somewhat unusual for a nuclear protein, and tends to suggest that it is not involved in a direct association with nucleic acids.

An extensive view of the literature has failed to discover any protein that demonstrates all of these characteristics. DNA polymerases catalyse the incorporation of deoxyribonucleotide monophosphates into a growing DNA chain via hydrolysis of the α - and β -phosphate groups. However, this reaction is dependent on the polymerase initially binding DNA, with hydrolysis to the dNMP moiety as a secondary step. It is also difficult to conceive of a polymerase showing binding affinity for its ligand that would preclude release. The DNA helicases show broad substrate specificity for their energy source, but are able to hydrolyse ATP more readily than dATP or other ribonucleotides and deoxyribonucleotides [14]. Similarly simian-virus-40 large T antigen has a broad substrate specificity, but with a preference for ATP and, more importantly, is not found in yeast. The DNA ligase enzymes use ATP, with the enzyme binding its ligand, cleaving the terminal two phosphate groups and transiently binding covalently to the 5'-phosphate of the DNA in an enzyme-AMP-DNA complex, activating the phosphate group and allowing ligation to occur. The effect of various deoxy- and ribonucleotides on the mobility of several transcription factor-Major Late Promoter-DNA complexes in gel retardation assays has been investigated [15]. These authors reported one dATP-specific change in the distribution of complexes in the assay, but there appears to be no correlation between transcription factor-Major Late Promoter-DNA binding and the protein-ligand interaction reported here. A requirement for ATP has been reported [16] for the association of nucleoplasm and HMG1 (high-mobility-group protein 1), two proteins found in the chromatin. This requirement, however, could be equally well fulfilled by dGTP, dUTP and, to a lesser extent, dCTP and dADP, though a non-hydrolysable analogue of ATP would also promote association,

suggesting it was not a requirement for energy. Ribonucleotide reductase can be discounted on the basis of its exclusive cytoplasmic location; it is able to hydrolyse ATP, the allosteric effector site will bind dATP, dGTP and dTTP, but not dCTP, and its main catalytic activity is the reduction of ribonucleotide diphosphates to the deoxy form [17].

We have good evidence that the protein or protein complex binds dATP and cleaves the terminal phosphate group. When considered with its stringent specificity for the deoxyribonucleotides and in the light of the background of eukaryotic nuclear enzymology presented, we conclude that we have isolated a novel activity operating in eukaryotes.

The high affinity of the protein for its ligand suggests that the bound nucleotide would be unavailable for activities such as repair or replication. It is therefore unlikely that the protein simply acts as a dATP carrier within the nucleus, but is more likely to play a regulatory role. The depletion of the deoxynucleotide pool by this enzyme could in itself form a crude regulatory mechanism. However, it is perhaps more likely that the modified protein acts within a regulatory pathway. Modulation of protein function could result either from the allosteric or covalent binding of the dADP moiety. Alternatively the regulatory mechanism may involve the transfer of the phosphate on to a substrate protein, or possibly both mechanisms operate.

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