Metal-dependent nucleotide binding to the *Escherichia coli* rotamase SlyD

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Upon expression and purification of the first catalytic domain of mammalian adenylate cyclase type 1 (IC1), a 27 kDa contaminant was observed, which was labelled by three radioactive ATP analogues (8-azido-ATP, 3'-O-(4-benzoyl)benzoyl-ATP and 2',3'-dialdehyde-ATP); the protein was purified separately and identified as *Escherichia coli* SlyD by N-terminal amino acid sequence determination. SlyD is the host protein required for lysis of *E. coli* upon infection with bacteriophage ΦX174 and has recently been shown to display rotamase (peptidylproline *cis*–*trans*-isomerase) activity. The covalent incorporation of ATP analogues into SlyD was promoted by bivalent transition metal ions ($Zn^{2+} \geq Ni^{2+} > Co^{2+} > Cu^{2+}$) but not by Mg^{2+} or Ca^{2+} ; this is consistent with the known metal ion specificity of SlyD. ATP, ADP, GTP and UTP suppressed labelling of SlyD with comparable potencies. Similarly, SlyD bound 2',3'-O-(2,4,6-trinitrophenyl)-ATP with an affinity in the range of 10 μM, as

determined by fluorescence enhancement. This interaction was further augmented in the presence of Zn^{2+} ($K_d = \sim 2 \mu M$ at saturating Zn^{2+}) but not of Mg^{2+} . Irrespective of the assay conditions, hydrolysis of nucleotides by SlyD was not detected. Upon gel filtration on a Superose HR12 column, SlyD (predicted molecular mass = 21 kDa) migrated with an apparent molecular mass of 44 kDa, indicating that the protein was a dimer. However, the migration of SlyD was not affected by the presence of Zn^{2+} or of Zn^{2+} and ATP. Thus we concluded that SlyD binds nucleotides in the presence of metal ions. These findings suggest that SlyD serves a physiological role that goes beyond that accounted for by its intrinsic rotamase activity, which is observed in the absence of metal ions.

Key words: ATP analogues, covalent incorporation, fluorescence enhancement, transition metal ions.

INTRODUCTION

Peptidyl–proline bonds in proteins can exist in two conformations (*cis* or *trans*). It has been appreciated for many years that mammalian organisms are endowed with enzymic activity that catalyses the change between the two conformations; the enzyme was originally purified from porcine kidney and was referred to as peptidylproline *cis*–*trans*-isomerase (PPIase) or 'rotamase' [1]. Subsequently, the rotamase activity was shown to reside in a cyclophilin [2,3]. The cyclophilin family members bind the immunosuppressant cyclosporin A with high affinity [4] and this interaction inhibits rotamase activity (for review see [5,6]). A different class of small proteins bind the immunosuppressant tacrolimus (FK506) and are therefore referred to as FK506 binding proteins. Similarly to cyclophilins, FK506 binding proteins display rotamase activity although there are neither sequence homologies nor similarities in folding topology between the two classes of proteins [6,7]. The *cis*–*trans* isomerization of peptidyl–proline bonds is thought to be one of the rate-limiting events in protein folding, hence rotamase activity is presumably involved in accelerating conformational transitions in folding intermediates. Both cyclophilins and FK506 binding proteins have been shown to accelerate folding of some proteins *in vitro* [5,6,8,9] and *in vivo* [10,11].

Because the activation barrier for the *cis*–*trans* rotation of the peptidyl–proline bond imposes an upper limit on the spontaneous folding rate of proteins, it was to be anticipated that PPIases were also present in prokaryotes; this was substantiated by the discovery of several bacterial rotamases, including the *E. coli* proteins rotA [12], parvulin [13], trigger factor [14] and SlyD [15,16]. SlyD confers sensitivity to lysis by bacteriophage ΦX174

(hence the name) and consists of two independent structural domains. The N-terminal domain (~150 amino acids) shows homology with FK506 binding proteins and SlyD was therefore classified as rotamase although PPIase activity was originally not detected using the standard chymotrypsin-based assay [15]. However, if trypsin was employed for product detection in the coupled assay, a peptidylproline *cis*–*trans*-isomerase activity of SlyD was observed, although the catalytic efficiency was considerably less than that of other PPIases [17]. The C-terminus of SlyD (~50 amino acids) contains many histidines grouped in clusters and this high histidine (and cysteine) density in SlyD supports high affinity binding of bivalent cations, in particular Ni^{2+} and Zn^{2+} [15], even in the presence of 6 M guanidine hydrochloride; hence the protein was also referred to as wondrous histidine-rich protein (WHP) [15]. In the present work, we have identified SlyD as a nucleotide binding protein; nucleotide binding (as assessed by covalent labelling with three different ATP analogues and by fluorescence enhancement using 2',3'-O-2,4,6-trinitrophenyl (TNP)-ATP) is enhanced in the presence of transition metal ions (in particular Zn^{2+}). Finally, we also show that SlyD is a dimer; dimerization, however, was not affected by the absence or presence of nucleotides and metal ions.

MATERIALS AND METHODS

Materials

[α -³²P]ATP and [γ -³²P]ATP were from Dupont NEN (Boston, MA, U.S.A.), 8-azido (⁸N₃)-[α -³²P]ATP and unlabelled 8-azido-ATP were from ICN (Irvine, CA, U.S.A.); ATP was purchased from Boehringer Mannheim (Mannheim, Germany); 4-benzoyl-

Abbreviations used: ⁸N₃, 8-azido; oATP, 2',3'-dialdehyde-ATP; Bz₂-ATP, 3'-O-(4-benzoyl)benzoyl-ATP; DTT, dithiothreitol; TNP, 2',3'-O-2,4,6-trinitrophenyl; IC1, the first intracellular domain of type-1 mammalian adenylate cyclase; WHP, wondrous histidine-rich protein; NTA, nitriloacetic acid.

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benzoic acid and 1,1'-carbonyldi-imidazole, 2-mercaptoethanol, adenosine and ddA were from Sigma-Aldrich (St. Louis, MO, U.S.A.). TLC plates, CNBr, *N,N*-dimethylformamide, NaIO₄, NaCNBH₃ and NaBH₄ were from Merck (Darmstadt, Germany); dithiothreitol (DTT) was from Biomol (Munich, Germany). Sephadex LH20, Sephadex G10 and Mono-Q were from Pharmacia (Uppsala, Sweden). Ni²⁺-nitriloacetic acid (NTA)-agarose was from Qiagen (Hilden, Germany). For autoradiography, X-Omat XAR5 (Kodak) and NEF 485 (NEN) films were used. TNP-ATP and TNP-ADP were from Molecular Probes (Leiden, The Netherlands). Reagents for electrophoresis were from Bio-Rad (Richmond, CA, U.S.A.).

Synthesis and purification of ATP analogues

ATP was oxidized with periodate to obtain 2',3'-dialdehyde ATP (oATP) according to Easterbrook-Smith et al. [18] and purified as described previously [19]. 3'-*O*-(4-benzoyl)benzoyl-ATP (Bz₂-ATP) was synthesized according to the method of Williams and Coleman [20] and the corresponding α -³²P-labelled analogues were synthesized in a similar manner using [α -³²P]ATP as starting material.

Purification and identification of SlyD

E. coli BL21DE3 was grown to a D_{600} of 1.4, the bacteria were harvested by centrifugation (5 min at 5000 *g*) and the bacterial pellet was resuspended in 50 mM Tris/HCl (pH 8)/1 mM 2-mercaptoethanol/0.1 mM PMSF (the equivalent of 1 litre of culture in 50 ml of buffer) and frozen in liquid N₂. After thawing, the cells were lysed by the addition of lysozyme (20 mg/l of original culture for 30 min, on ice). NaCl (final concentration 100 mM) was added to the soluble supernatant, obtained by centrifugation at 100000 *g* for 1 h, and was applied to an Ni²⁺-NTA-agarose column (300 ml of supernatant on to a 15 ml bed volume) pre-equilibrated in lysis buffer [50 mM Tris/HCl (pH 8.1), 1.0 mM 2-mercaptoethanol, 0.1 mM PMSF containing 100 mM NaCl]. The column was washed with 80 ml each of lysis buffer containing 100 mM NaCl, lysis buffer containing 500 mM NaCl, lysis buffer containing 100 mM NaCl and lysis buffer containing both 100 mM NaCl and 10 mM imidazole/HCl (pH adjusted to 7.4). Bound proteins were eluted by the addition of 100 mM imidazole/HCl, pH 7.4/1 mM 2-mercaptoethanol/0.1 mM PMSF/100 mM NaCl. The eluate was concentrated by ultrafiltration using an Amicon PM 10 membrane and the buffer was exchanged by repeated cycles of concentration and dilution with 20 mM Tris/HCl, pH 8.0/1 mM EDTA/1 mM DTT (overall dilution > 100-fold). The concentrated proteins were applied to a Pharmacia Mono-Q HR5/5 column equilibrated with 20 mM Tris/HCl, pH 8.0/1 mM EDTA/1 mM DTT, and the column was washed with 10 ml of buffer containing 200 mM NaCl (which eluted most of the proteins). The bound proteins were eluted with 30 ml of a linear gradient of 200–500 mM NaCl in 20 mM Tris/HCl, pH 8.0/1 mM EDTA/1 mM DTT and 1 ml fractions were collected. The absorbance was recorded at 280 nm. SlyD eluted as a symmetrical peak at ~ 340 mM NaCl. The appropriate fractions were pooled and concentrated in a Centricon 10 micro-concentrator. NaCl was removed by repeated cycles of concentration and dilution with 20 mM Tris/HCl, pH 8.0/1 mM EDTA/1 mM DTT and the protein, at a concentration of 2–4 mg/ml, was frozen in liquid N₂ and stored at –80 °C.

Analytical gel filtration was carried out using a Pharmacia Superose HR12 column; the buffer was 20 mM Hepes/NaOH, pH 7.0/0.1 M NaCl with or without Zn²⁺, ATP or both, as indicated in the Figure legends. The column was calibrated using

IgG (320 μ g), β -lactoglobulin (550 μ g), cytochrome C (300 μ g) and cyanocobalamin (vitamin B₁₂; 50 μ g); the migration of these molecular-mass markers was not affected by the presence or absence of Zn²⁺ or ATP, and the elution position of the standards was similar whether they were applied individually or in combination. The elution volume was calculated from the absorption at 280 nm. The protein samples (0.5 ml) were applied and 1 ml fractions were collected (flow rate 0.5 ml/min). The identity of the proteins in the UV-absorbance peaks was confirmed by SDS/PAGE.

Covalent labelling of SlyD with ATP analogues

Purified SlyD (0.5–3 μ g) was incubated, in the dark, for 1 h at 20 °C in 10–20 μ l buffer (20 mM Hepes/NaOH, pH 7.0, with EDTA and metal ion concentrations as indicated in the Figure legends) containing 80 μ M Bz₂-[α -³²P]ATP (specific radioactivity ~ 100–500 c.p.m./pmol), 100 μ M o-[α -³²P]ATP (specific radioactivity ~ 100–500 c.p.m./pmol) or 4–100 μ M ⁸N₃-[α -³²P]ATP (specific radioactivity ~ 200–4000 c.p.m./pmol); specific activities were adjusted by the addition of the unlabelled analogues. Thereafter, covalent incorporation of o-[α -³²P]ATP was achieved by the sequential addition of 100 mM NaCNBH₃ (1 min, on ice) and 10 mM NaBH₄ (10 min, on ice) [21]. For the incorporation of Bz₂-[α -³²P]ATP and ⁸N₃-[α -³²P]ATP, the samples were irradiated for 1 min, on ice, with a 100 W UV lamp placed at a distance of 5 cm above the reaction tubes. After the addition of Laemmli sample buffer, the samples were boiled and applied to SDS/13 % polyacrylamide gels. The incorporated radioactivity was detected by autoradiography (Kodak X-Omat; exposure time 12–24 h at –80 °C).

Fluorescence measurements

Fluorescence spectra were recorded (Hitachi F-4500) as described previously [22]; samples were contained in 0.2–0.25 ml buffer (20 mM Hepes, pH 7.0/1 mM EDTA) and the concentrations of SlyD, TNP-ATP or TNP-ADP, ATP, GTP, UTP, adenosine and ZnSO₄ are indicated in the Figure legends.

Miscellaneous procedures

The protein concentration was determined by dye binding with Coomassie Blue (Bio-Rad protein assay kit), using BSA as standard. The molar concentration of SlyD was calculated from the protein concentration assuming a molecular mass of 21 kDa. Potential ATPase (or apyrase) activity of SlyD was assayed by incubating SlyD (4–10 μ g) in 50 mM Hepes/NaOH, pH 7.5/1 mM EDTA/0.1 mM [γ -³²P]ATP (specific radioactivity, 5000 c.p.m./pmol) and 10 mM MgCl₂ or 2 mM ZnCl₂, or both. After 20 min at 20 °C, the reaction was stopped by the addition of 780 μ l ice-cold activated charcoal (5 % suspension in 50 mM NaH₂PO₄) to adsorb the nucleotides. The charcoal was sedimented and the radioactivity in a 400 μ l aliquot of the supernatant was estimated. The proteins were denatured in sample buffer containing 20 mM DTT, and SDS/PAGE was performed using Laemmli running buffer. The separated proteins were revealed by staining with Coomassie Blue and were transferred on to Immobilon P membranes, visualized by staining with Ponceau S and subjected to N-terminal sequencing by Edman degradation (Applied Biosystems 477A liquid-phase sequencer with an online HPLC system for phenylthiohydantoin-derivative analysis).

RESULTS

Covalent labelling of SlyD with ATP analogues

During the isolation of hexahistidine tagged IC1 (the first intracellular domain of mammalian type 1 adenylate cyclase [23,24]) from bacterial lysates, a 27 kDa contaminant was noted. This protein was labelled using three ATP analogues that differed with respect to the position and the nature of the reactive group (Figure 1). Covalent incorporation of oATP was achieved by reducing the Schiff's base formed between the reactive aldehyde groups of oATP and the ϵ -amino group of lysine residues of the protein with NaBH₄. Bz₂-[α -³²P]ATP and ⁸N₃-[α -³²P]ATP were modified on the ribose moiety and on the purine ring respectively.

Figure 1 Covalent labelling of SlyD by ⁸N₃-[α -³²P]ATP, Bz₂-[α -³²P]ATP and o-[α -³²P]ATP (A) and ⁸N₃-[α -³²P]ATP-labelling of SlyD in the presence of bivalent metal ions (B and C)

(A) IC1 and SlyD (4 μ g of total protein) were incubated in 10 μ l buffer (20 mM Hepes/NaOH, pH 7.5/1 mM EDTA/10 mM MgCl₂) containing 20 μ M ⁸N₃-[α -³²P]ATP (2000 c.p.m./pmol), 80 μ M Bz₂-[α -³²P]ATP (500 c.p.m./pmol) and 100 μ M o-[α -³²P]ATP (500 c.p.m./pmol) for 1 h in the dark at 20 °C. Covalent incorporation was achieved by irradiating the samples containing ⁸N₃-[α -³²P]ATP (8'-N₃-ATP) and Bz₂-[α -³²P]ATP (Bz-ATP) with UV-light for 1 min, on ice, or by the sequential addition of NaCNBH₃ and NaBH₄ to the sample containing o-[α -³²P]ATP (o-ATP), as described in the Materials and methods section. The proteins were resolved by SDS/PAGE and visualized by staining with Coomassie Blue. The incorporated radioactivity was detected by autoradiography (Kodal X-Omat, 12 h exposure at -80 °C). (B) SlyD (0.5 μ g) was incubated with 20 μ M ⁸N₃-[α -³²P]ATP in the presence of 0.1 mM or 1 mM of the indicated cation (divalent metal ion) in 10 μ l buffer (20 mM Hepes/NaOH, pH 7.5) for 1 h at 20 °C and irradiated with UV-light for 1 min. Sample buffer was added and samples were separated by SDS/PAGE. (C) SlyD (2.4 μ g) was incubated with 200 μ M ⁸N₃-[α -³²P]ATP in the presence of increasing concentrations of MgCl₂ or ZnSO₄ in 10 μ l buffer (20 mM Hepes/NaOH, pH 7.5/1 mM EDTA) for 1 h at 20 °C. Samples were irradiated with UV-light (1 min) and products were separated by SDS/PAGE. The Figure is representative of three experiments with different protein preparations. This experiment was repeated twice with similar results.

Figure 2 Inhibition of ⁸N₃-[α -³²P]ATP labelling of SlyD by nucleotides

SlyD (0.5 μ g) was incubated with 4 μ M ⁸N₃-[α -³²P]ATP in the presence of the indicated nucleotides in 20 μ l buffer (20 mM Hepes/NaOH, pH 7.5/1 mM ZnSO₄) for 1 h in the dark at room temperature. The samples were subsequently irradiated for 1 min with UV-light, on ice, as described in the Materials and methods section. The products were separated by SDS/PAGE and bands were visualized by staining with Coomassie Blue. The incorporated radioactivity was detected by autoradiography (Kodak X-Omat, 12 h exposure at -80 °C). The results are representative of three experiments with different protein preparations.

UV-photolysis generated a radical that allowed covalent labelling. It is evident from Figure 1 that the ATP analogues were more readily incorporated into the 27 kDa contaminant than into IC1, which comprises part of the catalytic ATP binding site of adenylate cyclase [25]. In order to identify the contaminant, the 27 kDa protein was therefore purified from untransformed bacteria and subjected to N-terminal sequencing. The amino acid sequence determined in the first 23 cycles was MKVAKDLV-VSLAYQVRTEDGVLV, which was 100 % identical with the N-terminus deduced for SlyD [16] and for WHP [15]. We stress that the predicted molecular mass of SlyD/WHP is 21 kDa but, on SDS/PAGE, the protein migrated with an apparent molecular mass of 27 kDa [15].

Metal-ion requirements for the incorporation of ATP into SlyD

Inspection of the deduced primary sequence for motifs found in nucleotide binding proteins revealed the presence of glycine-rich stretches in the C-terminus that are reminiscent of a Walker A-type motif [26], in particular G¹⁸⁰GEGCCGKG¹⁹⁰ in SlyD. Similarly, the sequence H¹⁴⁹GHVHGA¹⁵⁶ of SlyD may be related to the histidine triad H ϕ H ϕ H ϕ (where ϕ denotes a hydrophobic amino acid) that has recently been recognized as a nucleotide binding motif in a mammalian protein [27,28]. Most importantly, the ability of the histidine-rich sequence of SlyD to bind tightly to Ni²⁺-NTA-agarose and direct binding assays [16] indicates that the protein interacts with transition metal ions. Among the metal ions tested previously, Zn²⁺ was found to bind tightly to SlyD, Cu²⁺ and Co²⁺ bound to a lesser extent, and no appreciable interaction with Ca²⁺ was detected [16]. Consistent with this preference for metal ions, we observed that Zn²⁺ was most efficacious in promoting the incorporation of ⁸N₃-[α -³²P]ATP into SlyD; labelling was substantially lower in the presence of Cu²⁺ and Co²⁺ and was undetectable in the presence of Ca²⁺.

Figure 3 Fluorescence spectra of SlyD and TNP-ATP in the presence and absence of Zn²⁺ (A) and Zn²⁺ induced change in the fluorescence of SlyD and TNP-ATP (B)

(A) The excitation monochromator was set at 408 nm, the emission monochromator was changed at a rate of 200 nm/sec. The spectra were recorded sequentially in 200 μ l buffer (20 mM HEPES/NaOH, pH 8.0/1 mM EDTA). Trace 1, buffer; trace 2, SlyD (16 μ M); trace 3, TNP-ATP (42 μ M); trace 4, SlyD (16 μ M) + TNP-ATP (42 μ M); trace 5, SlyD + TNP-ATP + 1 mM ZnSO₄; trace 6, SlyD + TNP-ATP + 1 mM ZnSO₄ + 10 mM ATP. (B) The increase in fluorescence was measured by adding increasing concentrations of Zn²⁺ to 4 μ M SlyD and 14 μ M TNP-ATP. The excitation and emission wavelengths were 408 nm and 540 nm respectively. The results are representative of three independent experiments. AU, arbitrary units.

(Figure 1B). A similar metal-ion specificity was observed when SlyD was labelled with o-[α -³²P]ATP, and the efficacy of Ni²⁺ was comparable with that of Zn²⁺ (results not shown). The initial experiments, depicted in Figure 1(A), were carried out in the presence of Mg²⁺ because MgATP is the adenylate cyclase substrate. We therefore compared the ability of Mg²⁺ and Zn²⁺ to promote the incorporation of ⁸N₃-[α -³²P]ATP (Figure 1C) and of o-[α -³²P]ATP (results not shown) into SlyD. Zn²⁺ strongly promoted labelling, if the total metal ion concentration exceeded the sum of the chelating anions (EDTA and ⁸N₃-ATP). In contrast, concentrations of Mg²⁺ up to 10 mM had only a very modest effect on the labelling reaction and, furthermore, Mg²⁺ had no effect on the incorporation of ⁸N₃-[α -³²P]ATP promoted

by a saturating concentration of Zn²⁺ (Figure 1C, lane B). Taken together, these observations suggested that the protein bound Zn²⁺-ATP (as opposed to Zn²⁺-promoted binding of MgATP).

Inhibition of ⁸N₃-[α -³²P]ATP labelling of SlyD by nucleotides

When SlyD was incubated in the presence of a low concentration of ⁸N₃-[α -³²P]ATP (4 μ M), the covalent labelling of the protein was suppressed by ATP and ADP with a half-maximum inhibition in the range of approx. 20–30 μ M (Figure 2, upper panel). However, the labelling was not selective for adenine nucleotides because GTP and UTP also blocked the incorporation of the photoaffinity ligand (Figure 2, lower panel). Substantial suppression of labelling was seen at 50 μ M ATP, ADP and GTP, i.e. at a nucleotide concentration where the variation in free Zn²⁺ was trivial. We therefore rule out the possibility that the inhibition merely reflects the ability of the nucleotides to complex Zn²⁺. Analogous findings were obtained if ⁸N₃-[α -³²P]ATP was replaced by o-[α -³²P]ATP (results not shown). Labelling does not reflect phosphorylation of the protein, because no radioactivity was incorporated when SlyD was incubated with [γ -³²P]ATP. Finally, no hydrolytic activity was detected and no radioactivity was released from [γ -³²P]ATP, even when large amounts of the protein were used, irrespective of whether the assay was done in the presence of Zn²⁺, Mg²⁺ or a combination of these ions (results not shown).

Fluorescence enhancement of TNP-ATP by SlyD

As is evident from Figure 1, SlyD was covalently labelled by Bz₂-ATP, which shows that the protein was able to bind nucleotides with bulky substitutions on the ribose ring. This property was exploited to assess the Zn²⁺-requirement of nucleotide binding in a more quantitative manner by employing the fluorescent analogue TNP-ATP. When excited at 408 nm in aqueous solution, TNP-ATP emits a low-intensity fluorescence with a broad peak at 550–560 nm. Emission of fluorescence is greatly augmented in a hydrophobic environment, e.g. in a less polar solvent or when TNP-ATP is bound to a protein, and the emission peak is slightly blue-shifted to 540 nm [29–31]. Representative spectra are shown in Figure 3(A). SlyD *per se* was essentially devoid of any fluorescence [compare trace 2 and trace 1 (buffer control) in Figure 3A], regardless of the presence or absence of Zn²⁺ and/or Mg²⁺. However, the fluorescence of TNP-ATP (Figure 3A, trace 3) was augmented (and blue-shifted) if SlyD (Figure 3A, trace 4) was added to the cuvette. A further pronounced increase was seen upon addition of 1 mM Zn²⁺ to the combination of SlyD and TNP-ATP (Figure 3A, trace 5). This effect was not mimicked by Mg²⁺ (results not shown; see below and Figure 4), and, in the absence of SlyD, Zn²⁺ did not alter the fluorescence spectrum of TNP-ATP (results not shown). The fluorescence enhancement observed in the presence of TNP-ATP, SlyD and Zn²⁺ was quenched by the addition of ATP (Figure 3A, trace 6), indicating that TNP-ATP interacts with a nucleotide-binding site on SlyD (see below and Figure 5).

The fluorescence enhancement induced by Zn²⁺ was used to estimate the affinity of TNP-ATP-liganded SlyD for the metal ion by employing a constant concentration of TNP-ATP (14 μ M) and of SlyD (4 μ M). This yielded a saturation curve, and the half-maximal fluorescence was 10 ± 1.12 μ M total Zn²⁺ (Figure 3B). The affinity of Zn-ATP for SlyD was determined by recording the fluorescence of increasing concentrations of TNP-

Figure 4 Saturation of the SlyD-dependent increase in TNP-ATP fluorescence in the presence of 0.1 mM ZnSO₄ (A) and estimation of the K_d of TNP-ATP at various Zn²⁺ concentrations (B)

(A) SlyD (8 μM) was incubated with the indicated concentrations of TNP-ATP in 250 μl buffer (50 mM Hepes/NaOH, pH 7.0) containing 100 μM Zn²⁺. The inset shows the fluorescence recorded at 540 nm in the absence (○) or presence (●) of SlyD upon excitation at 408 nm. The difference between the two curves (▲) was plotted against the TNP-ATP concentration. (B) Saturation experiments were carried out as described in (A) at the indicated concentrations of SlyD and in the presence of 50 μM (▼), 100 μM (▲) or 1 mM (■) Zn²⁺; in the absence of Zn²⁺, the apparent K_d (◇) or in the presence of 2 mM Mg²⁺ (◆). The K_d (K_D) values were calculated by fitting the SlyD-dependent fluorescence enhancement to a rectangular hyperbola and plotting against the SlyD concentration. Data are means ± S.D. of two or three independent experiments.

ATP in the presence of 100 μM Zn²⁺; the difference between the fluorescence observed in the absence and presence of 8 μM SlyD yielded a saturation curve (Figure 4A). In the lower concentration range of TNP-ATP, SlyD was present in molar excess or in equimolar amounts, hence, the apparent K_d calculated from the saturation curve is inherently imprecise, because the depletion of free TNP-ATP is not taken into account. Accordingly, if saturation experiments were carried out at different concentrations of SlyD, the apparent K_d decreased in a linear manner with increasing concentrations of SlyD (Figure 4B). Depletion of TNP-ATP, however, can be corrected for by extrapolating the curve to intercept the y-axis, which will yield an estimate of K_d at infinitely low concentrations of SlyD. This gave a K_d of 1.7 ± 0.2 μM at 1 mM Zn²⁺ (Figure 4B, closed squares). A similar approach was used to estimate the K_d at lower Zn²⁺ concen-

Figure 5 Quenching of the fluorescence of TNP-ATP + SlyD by nucleotides in the absence or presence of Zn²⁺

SlyD (open symbols, 20 μM; closed symbols, 8 μM) was incubated in 250 μl buffer (50 mM Hepes/NaOH, pH 7.0) containing TNP-ATP (open symbols, 70 μM; closed symbols, 28 μM) in the absence (open symbols) or presence (closed symbols) of 1 mM Zn²⁺; nucleotide triphosphates (NTP), ATP (○, ●), GTP (△, ▲) or UTP (■), were added to give the indicated concentrations. The excitation and emission wavelengths were 408 nm and 540 nm respectively. The 100% control values (no added nucleotide) correspond to the difference in TNP-ATP fluorescence recorded in the absence or presence of SlyD; this was 220 and 180 arbitrary units for recordings in the presence or absence of Zn²⁺ respectively. Data are representative of three independent experiments.

trations and the K_d did not change or increased only marginally if the Zn²⁺ concentration was lowered to 100 μM (Figure 4B, closed triangles) or 50 μM Zn²⁺ (Figure 4B, closed inverted triangles). In contrast, in the absence of Zn²⁺ the affinity of TNP-ATP was substantially lower (Figure 4B, open diamond) and was not significantly affected by the presence of Mg²⁺ (Figure 4B, closed diamond).

Fluorescence quenching by nucleotides

As shown in Figure 3(A) (trace 6), an excess of ATP completely quenched the enhanced fluorescence resulting from the interaction of SlyD with TNP-ATP in the presence of 1 mM Zn²⁺. This was also observed in the presence of GTP and UTP, half-maximum inhibition being observed in the submillimolar range (Figure 5, closed symbols; $IC_{50} = 0.5 \pm 0.1$, 0.6 ± 0.1 and 0.7 ± 0.2 for GTP, ATP and UTP respectively). At these concentrations, it is, however, not possible to rule out that chelation of Zn²⁺ by the nucleotides contributes to fluorescence quenching. SlyD also binds TNP-ATP in the absence of Zn²⁺ (see the fluorescence enhancement by SlyD in Figure 3A, traces 3 and 4), albeit with a lower affinity (Figure 4B, open diamond). Therefore conditions in which a robust signal was obtained in the absence of Zn²⁺ were employed, i.e. the fluorescence in the presence of 20 μM SlyD and 70 μM TNP-ATP was recorded and the ability of ATP or GTP to quench the fluorescence was observed in the presence or absence of Zn²⁺. Half-maximum inhibition was seen with ATP and GTP (5.1 ± 1.8 mM and 4.9 ± 1.9 mM respectively; Figure 5, open symbols). TNP-ATP was present at about six times the K_d concentration range (see Figure 4B, open diamond); thus, using

Figure 6 Fluorescence enhancement of TNP-ADP and TNP-ATP by SlyD in the presence or absence of Zn^{2+}

SlyD (open symbols 10 μM ; closed symbols 4 μM) was incubated in 250 μl buffer (50 mM Hepes/NaOH, pH 7.0) containing the indicated concentrations of TNP-ADP (Δ , $\blacktriangle$) or TNP-ATP ($\circ$, $\bullet$) in the absence (open symbols) or presence (closed symbols) of 1 mM Zn^{2+} . The excitation and emission wavelengths were 408 nm and 540 nm respectively. Data are means $\pm$ S.D. from three independent paired experiments with three different preparations of SlyD.

the Cheng-Prusoff approximation $K_i = IC_{50}/[1 + A/K_A]$ (where K_i and K_A are the estimates of affinity of the inhibitor and the activator respectively, and A is the concentration of the activator), the IC_{50} values for ATP and GTP, in the absence of Zn^{2+} , produce an estimate of K_i of about 0.7 mM. We have also shown that, in control experiments carried out in dimethylformamide, the nucleotides *per se* do not quench the fluorescence of TNP-ATP (results not shown).

Comparison of TNP-ATP and TNP-ADP

ADP (Figure 2) and other nucleoside diphosphates (results not shown) suppress the covalent incorporation of ATP analogues into SlyD. This observation indicates that SlyD does not discriminate between ADP and ATP in the presence of Zn^{2+} . The ability of the protein to bind a nucleotide diphosphate was assessed by employing the analogue pair TNP-ADP or TNP-ATP (Figure 6). In the absence of Zn^{2+} , SlyD enhanced the fluorescence of TNP-ADP to a substantially lesser extent than that of TNP-ATP. In contrast, in the presence of Zn^{2+} , SlyD interacted with TNP-ADP and TNP-ATP with comparable affinity. The apparent K_d values were 2.8 ± 0.2 and 2.1 ± 0.3 μM for TNP-ADP and TNP-ATP respectively. We have also verified that similar concentrations of Zn^{2+} were required to promote the binding of TNP-ADP and TNP-ATP (results not shown). Taken together, these data indicate that SlyD discriminates between nucleoside diphosphates and triphosphates only in the absence of metal ions.

Gel-filtration chromatography of SlyD

In the initial attempts to resolve IC1 from SlyD we noted that the protein migrated with an apparent molecular mass that was larger than expected. This was verified by analytical gel filtration on a calibrated Superose HR12 gel-filtration column. The elution

Figure 7 Migration of SlyD on a Superose HR gel-filtration column

SlyD (approx. 200 μg) was applied on to a Superose 12 column using buffer with various additions. (A) Fractions (1 ml) were eluted from a Superose column with 20 mM Hepes/NaOH, pH 7.0/1 mM $ZnSO_4$ /100 μM $MgCl_2$ /100 mM NaCl and 20 μl of each fraction was applied on to an SDS-polyacrylamide gel. The separated proteins were stained with Coomassie Blue. (B) The column was calibrated with molecular mass markers, IgG, BSA, β -lactoglobulin, cytochrome c and cyanocobalamin. The eluate volume of each standard was plotted against the log of its molecular mass to produce the regression line. (∇) Elution of SlyD in buffer (20 mM Hepes/NaOH, pH 7.0/100 mM NaCl); (Δ) elution of SlyD in buffer containing 1 mM $ZnSO_4$; ($\circ$) elution of SlyD in buffer containing 3 mM $ZnSO_4$ and 2 mM ATP. The arrow ($\downarrow$) shows the calculated eluate volume of SlyD if the protein migrated as a monomer (21 kDa). Data are representative of three independent experiments.

profile of SlyD is shown in Figure 7(A), SlyD was eluted as a single band in two fractions with an apparent molecular mass of 44 kDa (Figure 7B). This is consistent with a dimeric complex formed by two molecules of SlyD (the predicted molecular mass being 21 kDa [15,16]). The presence of Zn^{2+} , ATP or a combination of both did not affect the migration of SlyD (Figure 7B).

DISCUSSION

The results obtained in the present work show that SlyD is a nucleotide binding protein which interacts equally well with purine or pyrimidine nucleotides but displays a strong preference for Zn^{2+} (and Ni^{2+}) over other transition metal ions or Mg^{2+} and Ca^{2+} . This conclusion is based on both the metal-ion-dependent covalent incorporation of three different ATP analogues into SlyD and the ability of SlyD to enhance the fluorescence of TNP-ATP. TNP-ATP bound to SlyD with substantially higher affinity than the naturally occurring nucleotides, both in the absence and

presence of Zn^{2+} ; e.g. in the absence of Zn^{2+} , the K_d of SlyD for TNP-ATP was estimated to be $10\ \mu M$, whereas that for ATP (or GTP) was calculated to be about $0.7\ mM$. However, in general, the affinity of TNP-ATP for ATP-binding sites was substantially higher (by two to three orders of magnitude) than that of the unmodified nucleotide [29–31]. Thus the difference in affinity estimates for TNP-ATP and nucleotide triphosphates observed in the present work is not without precedent. More importantly, the available evidence suggests that SlyD is a dimer, the formation of which was not affected by binding of Zn^{2+} or ATP. Although Zn^{2+} -dependent binding of nucleotides to proteins is unusual, it is not without precedent. The homodimeric *E. coli* protein MukB, which is required for correct partitioning of the bacterial chromosome to daughter cells, binds GTP and ATP in the presence of Zn^{2+} but not of Mg^{2+} . No appreciable difference in the affinity of MukB for ATP and GTP was observed; other nucleotides were not tested [32]. However, it is conceivable that MukB [32] and SlyD may hydrolyse bound nucleotide triphosphates upon interaction with appropriate reaction partners which provide the residues required for catalysis.

The precise physiological role of SlyD is not clear. SlyD was discovered as the bacterial gene product that is essential for the life cycle of the bacteriophage $\Phi X174$. *E. coli* strains deficient in SlyD are viable but they do not produce infectious phages and the mature phage accumulates in the bacterial cell. Lysis, which depends on the product of the $\Phi X174$ *E* gene [33] and on the presence of the host protein SlyD, does not occur [16]. Independently, SlyD was described as a WHP that was shown to bind Zn^{2+} and Ni^{2+} [15], and to have rotamase activity [17]. A role for SlyD in bacterial cell division was proposed based on the morphological changes occurring during cell lysis mediated by the *E* gene product [34,35], and those observed upon over-expression of the protein in bacteria [36]. This may be related to the rotamase activity of SlyD as there is a precedent for a role of eukaryotic rotamases in cell division [37,38].

However, a model in which the function of SlyD is related exclusively to its intrinsic rotamase activity does not account for the metal-ion-binding capacity specified by the C-terminus of SlyD. The C-terminal domain is responsible for both the ability of SlyD to confer sensitivity to lysis by the bacteriophage *E* protein [16,36] and for rotamase activity [17]. In fact, binding of metal ions to the C-terminus of SlyD inhibits rotamase activity [17]. The C-terminus of SlyD is related to the histidine-rich domains of bacterial HypB GTPases [39], which are responsible for generating active hydrogenases. In HypB, a large number of clustered histidine residues bind Ni^{2+} which is incorporated into the active site of the hydrogenase by a reaction driven by the hydrolysis of GTP [40]. HypB of *E. coli* is the only currently known isoform that lacks this histidine-rich domain but nevertheless displays GTPase activity [41]. It was therefore proposed that HypB of *E. coli* may co-operate with SlyD in the Ni^{2+} transfer reaction [17]. Our findings that SlyD binds nucleotides are in line with the hypothesis that the protein has a role beyond that of its rotamase activity. It should be noted also that the evidence that links the action of immunophilins on protein folding to their rotamase activity *in vivo* is indirect [10,11]. There are several examples where immunophilins exert their regulatory effects in a manner that is independent of their PPIase activity [42–44].

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