The *Escherichia coli* SlyD Is a Metal Ion-regulated Peptidyl-prolyl *cis/trans*-Isomerase*

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In *Escherichia coli* as many as nine different genes coding for proteins with significant homology to peptidyl-prolyl *cis/trans*-isomerases (PPIases) have been found. However, for three of them, the histidine-rich SlyD, the homologous gene product of ORF149, and parvulin-like SurA, it was not known whether these proteins really possess PPIase activity. To gain access to the full set of PPIases in *E. coli*, SlyD, the N-terminal fragment of SlyD devoid of the histidine-rich region, as well as the protein product of ORF149 of *E. coli* named SlpA (*SlyD-like protein*) were cloned, overexpressed, and purified to apparent homogeneity. On the basis of the amino acid sequences, both proteins proved to be of the FK506-binding protein type of PPIases. Only when using trypsin instead of chymotrypsin as helper enzyme in the PPIase assay, the enzymatic activity of full-length SlyD and its N-terminal fragment can be measured. For Suc-Ala-Phe-Pro-Arg-4-nitroanilide as substrate, k_{cat}/K_m of 29,600 M^{-1} s^{-1} for SlyD and 18,600 M^{-1} s^{-1} for the N-terminal fragment were obtained. Surprisingly, the PPIase activity of SlyD is reversibly regulated by binding of three Ni^{2+} ions to the histidine-rich, C-terminal region. Because the PPIase activity of SlpA could be established as well, we now know eight distinct PPIases with proven enzyme activity in *E. coli*.

Peptidyl-prolyl *cis/trans*-isomerases (PPIases,[1] EC 5.2.1.8.) are able to accelerate the *cis-trans*-isomerization of the Xaa-Pro peptide bond in polypeptide chains (1). They are divided into three families, the cyclophilins, FK506-binding proteins (FKBP) including the putative subfamily of trigger factors, and parvulins. Members of all three PPIase families have been found in *Escherichia coli* (2–6).

The *slyD* gene (*s*ensitivity to *ly*sis) was identified by screening for survival of *E. coli* C after induction of the cloned lysis gene E of bacteriophage φX174 (7). Mutations mapped to the *slyD* locus (at 73.5′ on the *E. coli* chromosome) resulted in accumulation of complete phages inside the cells, but no lysis occurred. The *slyD* locus was cloned and sequenced. By deletion analysis it was demonstrated that the open reading frame 196 of *E. coli* is identical to *slyD* (8). The derived amino acid sequence of SlyD was shown to share similarity with FKBPs. Independently, the protein SlyD (there called WHP, *w*onderous *h*istidine-rich *p*rotein) was discovered by binding to nickel ions immobilized on nitriloacetic acid-agarose (NTA) resin (9).

Derived from the amino acid sequence, SlyD consists of two sequence regions, an N-terminal stretch of 146 amino acids with 28.1% similarity to hFKBP12 and a C-terminal histidine-rich stretch. Similar regions containing clustered histidine residues are usually present in HypB proteins involved in nickel insertion into hydrogenases (10). Only HypB of *E. coli* lacks this histidine-rich stretch. In contrast to HypB of other species, it is not able to bind nickel ions (11). A hypothetical protein with a similar structure as SlyD was detected in *Haemophilus influenzae* (12).

The deduced amino acid sequence of the ORF149 from *E. coli* (13) is homologous to amino acids 1–146 of SlyD. A protein, perhaps derived from ORF149 of *E. coli*, was found earlier after cloning of a 7.1 kilobase insert generated by restriction of the specialized transducing phage λdapB2 with the restriction endonuclease *Bam*HI (14), and a 17-kDa protein was detected by coupled transcription and translation. After sequencing of the *lsp-dapB* interval (13), it was suggested that the 17-kDa protein is coded by ORF149. The ORF149 is part of the *ileS-lsp* operon (15). An operon of the same structure, *x-ileS-lsp-orf149-orf316*, was found in *Enterobacter aerogenes* (16). A similar ORF has also been reported for *Pseudomonas fluorescens* (17) and for *Methanococcus jannaschii* (18).

Here we describe that SlyD is indeed a PPIase. Its enzymatic activity can be reversibly inhibited by Ni^{2+} ions. Metal ion influence disappears for the N-terminal PPIase fragment of SlyD (amino acids 1–146) lacking the histidine-rich region. Just like SlyD, the related protein product of ORF149 of *E. coli* (SlpA) shows PPIase activity.

EXPERIMENTAL PROCEDURES

Cloning and Overexpression—All genes or part of the genes were amplified from *E. coli* strain BL21 chromosomal DNA by polymerase chain reaction (PrimeZyme, Biometra, Göttingen, Germany) and were ligated into the overexpression plasmid pQE30 (Qiagen, Hilden, Germany) followed by transformation into competent *E. coli* M15(pREP4) cells. The cells were selected on Luria-Bertani agar plates containing 50 μg/ml ampicillin and 10 μg/ml kanamycin. The plasmids of the obtained clones were examined by restriction analysis and DNA sequencing. In addition, the clones were screened for overexpression of a protein of the right size in small scale cultures. Three h after induction by 1 mM IPTG, the cells were pelleted by short centrifugation, dissolved in sample buffer, and analyzed by SDS-PAGE (19) and Coomassie Blue staining (20).

For cloning of the *slyD* gene, the oligonucleotide primers 5′-CGCC-

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[1] The abbreviations used are: PPIase, peptidyl-prolyl *cis/trans*-isomerase; FKBP, FK506-binding protein; NTA, nitriloacetic acid; ORF, open reading frame; IPTG, isopropyl β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis; MOPS, 4-morpholinepropanesulfonic acid; MES, morpholineoethanesulfonic acid; Suc, succinyl; 4NA, 4-nitroanilide; CsA, cyclosporin A; AES, atomic emission spectroscopy; HPLC, high pressure liquid chromatography.

AGAATTCTTCCCATGCTCAGG-3′ and 5′-GCGTCTGGGATCCGAGA-AGTTAAAAGCCAGCC-3′ were used. The obtained fragment was cloned into pQE30 by using the restriction endonucleases *Eco*RI and *Bam*HI. For cloning of the N-terminal fragment of SlyD (amino acids 1–146), the same cloning strategy as for SlyD with the above mentioned first primer and the primer 5′-GTGAAGGATCCTCAGCTAATTATTCT-TCTTCAGTCGC-3′ was used. Cloning of the ORF149 was achieved by polymerase chain reaction using the primers 5′-CGGCAAAGAATT-CTAAATATAAG AGC-3′ and 5′-CGCGAGCTCTCGACCACATAG-CG-3′ followed by ligation of the fragment into the *Eco*RI and *Sac*I restriction sites of pQE30. All DNA manipulations were performed according to Sambrook *et al.* (21). DNA sequencing of recombinant plasmids was performed by the Sanger method of chain termination using the T7 sequencing kit (Pharmacia, Freiburg, Germany) and the pQE type III/IV or reverse primers (Qiagen, Hilden, Germany). All DNA modifying enzymes were purchased from Boehringer Mannheim (Germany).

Overexpression of recombinant proteins was achieved by inoculating 1 liter of medium containing 50 μg/ml ampicillin and 10 μg/ml kanamycin with 20 ml of overnight culture of the appropriate strain and growing at 37 °C and 200 rpm shaking to an A_{600} of 0.5. After induction with 1 mM IPTG, the cells were grown for another 5 h. The cells were harvested at 4,000 $\times$ g for 10 min in a Beckman J2-HC centrifuge (Beckman Instruments, Palo Alto, CA) and dissolved in the buffer used for purification on the first column. For degradation of nucleic acids, 0.1% (v/v) benzonase was added. The cell suspension was passed 3 times through a French press cell (SLM Aminco, Buettelborn, Germany) at 20,000 p.s.i. After the removal of membrane debris by centrifugation at 95,800 $\times$ g for 40 min at 4 °C in a Beckman L8 60 M centrifuge, the supernatant was submitted to further purification.

Determination of Protein Concentration, SDS-PAGE, Western Blotting—For determination of the protein concentration, the method of Bradford (22) and the absorbance at 280 nm were used. The extinction coefficients ϵ at 280 nm were calculated according to Gill and van Hippel (23). Polyacrylamide gel electrophoresis was performed as described by Laemmli (19), and the protein bands were visualized by silver staining (24) or a modified Coomassie Blue staining method (20). Polyclonal antibodies against SlyD and SlpA were raised in rabbits (PAB productions, Hebertshausen, Germany) against proteins desalted by HPLC as described below. Western blotting to nitrocellulose was performed in a semidry electroblotting system (Fast Blot B32, Biometra, Göttingen, Germany) with a buffer containing 25 mM Tris, 150 mM glycine, 10% (v/v) methanol, pH 8.3. The membrane was incubated with diluted polyclonal antibodies (1:2,000) for 1 h at room temperature. For detection, the blot was incubated with peroxidase-conjugated goat anti-rabbit IgG (Sigma, Deisenhofen, Germany) and developed with 0.018% 4-chloro-1-naphthol in 50 mM Tris, 150 mM NaCl, 0.024% hydrogen peroxide, pH 6.0.

Purification of Recombinant Proteins—SlyD was purified by affinity chromatography on Ni^{2+}-NTA-agarose (Quiagen, Hilden, Germany) with a 0–100 mM imidazole gradient in 100 mM Tris, 1 M NaCl, pH 8.0. After dialysis against 10 mM MOPS, 1 mM dithiothreitol, pH 7.0, an anion exchange chromatography on Fractogel EMD DEAE-650(M) was performed. The SlyD containing fractions were saturated to 30% with ammonium sulfate and applied on a propyl-Sepharose column. The protein was eluted with a gradient of 30–0% saturation ammonium sulfate and dialyzed thoroughly against 10 mM MOPS, 1 mM dithiothreitol, pH 7.0. After that the protein was enriched on a DEAE column. About 1 mg of homogeneous SlyD was desalted by HPLC and used to generate antibodies in rabbit. All protein preparations were performed at 4 °C. Purification was examined by SDS-PAGE and Western blotting.

The N-terminal fragment of SlyD was purified by applying the bacterial lysate on a Fractogel EMD DEAE-650(M) column (Merck, Darmstadt, Germany) equilibrated with 50 mM Tris, pH 7.5. The protein eluted with a 0–2 M NaCl gradient and was passed in the same buffer through a column packed with chelating Sepharose Fast Flow (Pharmacia, Uppsala, Sweden). Beforehand, the column was loaded with NiSO$_4$ and washed with 50 mM Tris, pH 7.5. The protein fraction, which did not bind to the column, was dialyzed in 20 mM MES, pH 5.5, and passed through a Fractogel TSK AF-Blue column in the same buffer. The protein solution was concentrated on a Filtron Omegacell (exclusion size 10,000 Da). Samples of 1 ml were submitted to gel filtration on a Superdex 75 16/60 HiLoad FPLC column (Pharmacia, Uppsala, Sweden) equilibrated with 10 mM HEPES, 150 mM KCl, pH 7.8. After dialysis against 20 mM MES, pH 5.5, the appropriate fractions were applied in dialysis buffer on an EMD DEAE-650(M) column. The protein was eluted with a linear gradient from 0–2 M NaCl.

Purification of SlpA was achieved by anion exchange chromatography on Fractogel EMD DEAE-650(M) with 20 mM Tris, pH 7.5. The protein eluted with a linear gradient from 0–2 M NaCl and was passed through a chelating Sepharose Fast Flow column loaded with NiSO$_4$ and equilibrated with 20 mM HEPES, 300 mM NaCl, pH 7.0. After concentration on a Filtron Omegacell (exclusion size 10,000 Da) the protein was submitted to gel filtration on a Superdex 75 column, equilibrated with 10 mM HEPES, 150 mM KCl, pH 7.8. The protein was dialyzed against 20 mM MES, pH 5.5, and passed through a Fractogel TSK AF-Blue column.

Measurement of PPIase Activity—The protease coupled assay of Fischer *et al.* (1, 25) for determination of PPIase activity was used. Before any measurements were carried out, the protease stability of the possible PPIases was examined. About 100 μg/ml homogenous protein was incubated for either 20 s or 2 min in 35 mM HEPES, pH 7.8, at 10 °C with chymotrypsin (30 μM), subtilisin (2 μM), thrombin (60 μM), and trypsin (0.5 μM). The reaction was stopped by adding the serine protease inhibitor phenylmethanesulfonyl fluoride (1 mM final concentration) and boiling sample buffer. The samples were analyzed by SDS-PAGE and Western blotting.

For the determination of PPIase activity either 0.5 μM trypsin or 30 μM chymotrypsin and substrates of the structure Suc-Ala-Xaa-Pro-Arg-4NA and Suc-Ala-Xaa-Pro-Phe-4NA (Xaa = variable aminoacyl residue) were used, respectively. Stock solutions of substrates (10 mg/ml) were prepared in dimethyl sulfoxide. The reactions were carried out in 35 mM HEPES, pH 7.8, at 10 °C and followed spectrophotometrically at 390 nm using a Hewlett-Packard 8452 diode array UV/VIS spectrophotometer. 500 data points were used to calculate the first-order rate constants. Inhibition of PPIase activity was measured by adding FK506, CsA, or metal chlorides to the reaction mixture 5 min before starting the measurement. Stock solutions of 10 mM FK506 (gift from Fujisawa Pharmaceutical Co., Osaka, Japan) or 100 mM CsA in ethanol, 1 mM NiCl$_2$, or other metal chlorides (ZnCl$_2$, CaCl$_2$, NaCl, KCl, and MgCl$_2$) in 35 mM HEPES, pH 7.8, were used.

Analysis of the Nickel Ion Binding to SlyD—Equilibrium dialysis was performed in home-made cells (26) for 12 h in a humid chamber at 10 °C. The lower chamber contained 250 μl of the appropriate NiCl$_2$ solution in 35 mM HEPES, pH 7.8. The upper chamber was filled with 250 μl of SlyD solution (72 μM) in the same buffer.

The nickel content of SlyD and buffer samples was determined by inductively coupled plasma AES with a Jenaquant AES 110 (Zeiss, Jena, Germany). For calibration, 1-element standards (Merck, Darmstadt, Germany) and 35 mM HEPES, pH 7.8, as metal-free control have been used.

The data obtained by AAS were analyzed by means of the Adair equation (27).

$$r = \frac{K_1[\text{Me}] + 2K_1K_2[\text{Me}]^2 + 3K_1K_2K_3[\text{Me}]^3}{1 + K_1[\text{Me}] + K_1K_2[\text{Me}]^2 + K_1K_2K_3[\text{Me}]^3} \qquad \text{(Eq. 1)}$$

[Me] stands for the free metal concentration, r is the number of metal ions bound per mole of protein, and K_1, K_2, and K_3 are the binding constants of three different sites.

The data from the inhibition studies of the PPIase activity of SlyD by NiCl$_2$ were converted to binding data. The specific PPIase activity of SlyD samples without added NiCl$_2$, as these prepared for AES, should equal 0.24 bound nickel ions/molecule of SlyD (as determined by AES for these samples). All other data points were calculated, assuming linear dependence of nickel binding to inhibition of PPIase activity of SlyD.

CD Spectrometry of SlyD—Homogeneous samples of SlyD were applied to a desalting column (Econo-Pac® 10 DG, Bio-Rad, Hercules, USA). The protein was eluted with 5 mM phosphate buffer, pH 6.0. CD spectra were recorded from 190 to 260 nm with 12.5 μM SlyD. When required, 100 μM NiCl$_2$ (final concentration) was added 5 min before starting the measurement. All measurements were performed in a 1-mm cuvette at 25 °C with a J-710 spectropolarimeter (Jasco, Tokyo, Japan), averaging 20 transients with Jasco software Version 1,33.00.

HPLC, Mass Spectrometry and Protein Sequencing—Reversed-phase HPLC separations for antibody production, protein sequencing, and mass spectrometry were performed on a Shimadzu LC-10A modular HPLC unit (Shimadzu, Kyoto, Japan). The protein was applied to a C$_3$ column (125 $\times$ 4 mm Nucleosil 500–5 C$_3$-PPN, Macherey-Nagel, Düren, Germany) in 1% acetonitrile, 0.1% trifluoroacetic acid and eluted with a 30–60% acetonitrile gradient in 0.1% trifluoroacetic acid for 20 min at 40 °C (flow rate 1 ml/min). The molecular mass of proteins embedded in sinapic acid was determined using a MALDI-TOF Bruker reflex mass spectrometer (Bruker-Franzen-Analytik, Bremen, Germany) with a N$_2$-UV laser (337 nm). Amino acid sequences were determined using an Applied Biosystems 476A gas phase sequencer (Applied Biosystems, Foster City, USA).

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SlyD E.c.     1 ..........  ....MKVAKD LVVSLAYQVR TEDGVLVDES PVSA.PLDYL HGHGSLISGL
SlpA E.c.     1 ..........  ..MSESVQSN SAVLVHFTLK LDDGTTAEST RNNGKPALFR LGDASLSEGL
Tig E.c.    145 ...RKQQATW KEKDGAVEAE DRVTIDFTGS .VDGEEFEGG KASD..FVLA MGQGRMIPGF
hFKBP12 H.s.  1 MGVQVETISP GDGRTFPKRG QTCVVHYTGM LEDGKKFDSS RDRNKPFKFM LGKQEVIRGW
                                              *            **       *      *     *** *


SlyD E.c.    46 ETALEGHEVG DKFDVAVGA. NDAYGQYDEN LVQRVPKDVF MGVDELQVGM RFLAETDQGP
SlpA E.c.    49 EQHLLGLKVG DKTTFSLEPD AAFGVPSPDL IQYFSRREFM DAGEPEIGAI MLFTAMDGSE
Tig E.c.    199 EDGIKGHKAG EEFTIDVTFP EE.YHAE... .......... .......... ..........
hFKBP12 H.s. 61 EEGVAQMSVG QRAKLTISPD Y.AYGATGH. .......... .......... ..........
                                            **      *


SlyD E.c.   105 VPVEITAVED DHVVVDGNHM LAGQNLKFNV EVVAIREATE EE
SlpA E.c.   109 MPGVIREING DSITVDFNHP LAGQTVHFDI EVLEIDPALE A
Tig E.c.    229 .......... .........N LKGKAAKFAI NLKKVEEREL PELTAE
hFKBP12 H.s. 89 .PGIIPPHA. .......... ....TLVFDV ELLKLE
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FIG. 1. **Sequence alignment of the FKBP-like fragment of SlyD (*E. coli* (*E.c.*) accession number SwissProt (SP): P30856), SlpA (ORF149, *E. coli*, accession number SP: P22563), Trigger factor (Tig, *E. coli*, accession number Protein Identification Resource (PIR) 2 A36129), and the human FKBP12 (hFKBP12, *Homo sapiens* (*H.s.*), accession number PIR3: S11089).** Amino acids assumed to be essential for binding of FK506 to hFKBP12 (33) are marked by an *asterisk* below, and identical amino acids are printed in *bold letters*.

RESULTS

Purification of SlyD, the N-terminal Fragment of SlyD (Amino Acids 1–146) and SlpA—SlyD was purified from overexpression cultures with a yield of 54 mg of homogenous protein/liter of culture. By silver staining, only a single band could be detected in SDS-PAGE after the last purification step. Amino acid sequencing of the purified protein for 10 steps showed the expected N-terminal sequence MKVAKDLVVS of SlyD (Fig. 1). The N-terminal methionine was not processed. Analysis of the protein by mass spectrometry showed a molecular mass of 20,850 Da, very similar to the theoretical mass of 20,852 Da. The apparent molecular mass in SDS-PAGE was 25 kDa.

The purification of the N-terminal fragment of SlyD yielded 9 mg of homogenous protein/liter of culture. The protein did not bind to nickel ions immobilized on chelating Sepharose. In SDS-PAGE, the apparent molecular mass of the N-terminal fragment of SlyD was 18 kDa. Antibodies raised against the complete SlyD molecule were able to detect the N-terminal fragment of SlyD in Western blots.

The ORF149 of *E. coli* was cloned, and the published sequence (13) was verified. The protein product (SlpA) was purified to homogeneity as shown by SDS-PAGE and Coomassie Blue staining at a level of 17 mg/liter of culture. SlpA passed through a chelating Sepharose column preloaded with nickel ions. The measured molecular mass of 15,946 Da was in good agreement with the calculated value of 15,949 Da for SlpA if the methionine in position 1 has been cleaved off during processing. With antibodies generated against recombinant SlpA, a protein of the same apparent molecular mass as SlpA was detected in *E. coli* BL21.

PPIase Activity of SlyD and Related Proteins—In previous experiments using the standard assay coupled with chymotrypsin (1, 25), no PPIase activity of SlyD has been detected (9). We found that this is due to a high sensitivity of SlyD against chymotrypsin. After 2 min of incubation with 30 μM chymotrypsin or 2 μM subtilisin, SlyD was no longer detected in the Western blot. Even after 20 s of incubation, the amount of SlyD was considerably reduced. In contrast, SlyD remained stable for at least two min toward 0.5 μM trypsin and 60 μM thrombin. For that reason, PPIase activity measurements were carried out with trypsin, another isomer-specific protease. For the substrates Suc-Ala-Phe-Pro-Arg-4NA, Suc-Ala-Ala-Pro-Arg-4NA, and Suc-Ala-Leu-Pro- Arg-4NA, specificity constants k_{cat}/K_m of 29,600, 6,200, and 5,600 M^{-1} s^{-1} were measured. After addition of chymotrypsin (final concentration 30 μM) to the reaction mixture, no PPIase activity was detected for SlyD, explaining
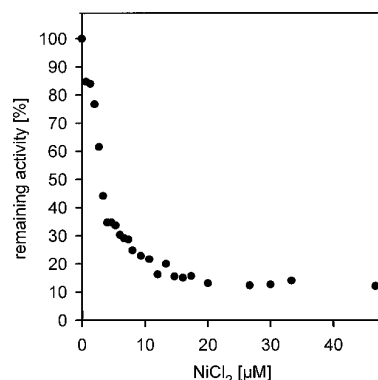

FIG. 2. **Inhibition of the PPIase activity of SlyD by NiCl$_2$.** Measurements were performed in 35 mM HEPES, pH 7.8, at 10 °C using trypsin and the substrate Suc-Ala-Ala-Pro-Arg-4NA. Concentration of SlyD was 1.09 μM. Each data point represents mean of three measurements. The k_{enz} value without NiCl$_2$ (6.68×10^{-3} s^{-1}) was set to 100%.

the failure to detect it in the standard assay.

As shown earlier (9), SlyD is able to bind Ni^{2+} and Zn^{2+} ions. The PPIase activity of SlyD was considerably reduced in a reversible manner by addition of NiCl$_2$ at micromolar concentrations (Fig. 2). Incubation of a sample of SlyD (1 μM) with 50 μM NiCl$_2$ decreased the PPIase activity of SlyD to 10%. The activity could be completely restored by dilution into nickel-free buffer. Other metal chlorides tested (CaCl$_2$, NaCl, KCl, MgCl$_2$) did not affect the PPIase activity up to 1 mM final concentration. However, it was not possible to examine the influence of ZnCl$_2$ due to the inactivation of trypsin in the protease coupled assay. The PPIase activity of SlyD was inhibited neither by 25 μM FK506 nor by 100 μM CsA, otherwise typical inhibitors of PPIases, in the reaction mixture.

The PPIase activity of the N-terminal fragment of SlyD measured using trypsin-mediated cleavage of the substrates Suc-Ala-Phe-Pro-Arg-4NA, Suc-Ala-Ala-Pro-Arg-4NA, and Suc-Ala-Leu-Pro-Arg-4NA was found to be 18,600, 3,900, and 3,600 M^{-1} s^{-1}, respectively. This activity was also not inhibited by 25 μM FK506 or 100 μM CsA. No inhibition of PPIase activity was detected up to 100 μM NiCl$_2$. The N-terminal fragment of SlyD showed the same sensitivity to chymotrypsin as the complete protein.

The purified protein SlpA was tested for PPIase activity in the chymotrypsin coupled assay. In contrast to SlyD, SlpA remained active even after 2 min of incubation with chymotrypsin. The protease resistance was also confirmed in SDS-

All measurements were carried out in 35 mM HEPES, pH 7.8, at 10 °C (cyclophilins, 15 °C) with chymotrypsin and substrates of the structure Suc-Ala-Xaa-Pro-Phe-4NA. For calculation of the percentage specific PPIase activity, the k_{cat}/K_M value for Xaa = Phe was set to 100%. The data for FKBP22, parvulin, trigger factor, and both cyclophilins were taken from literature. ND, not determined.

Xaa	SlpA	FKBP22 (4)	Parvulin (5)	Trigger factor (6)	Cyp21 (2)	Cyp18 (2)
Phe	100	100	100	100	100	100
Leu	83	205	120	58	192	120
Ile	43	12	40	3	ND	ND
Lys	38	53	30	23	39	17
Ala	37	6	60	22	400	346
Trp	25	21	44	10	ND	ND
His	21	19	34	28	28	55
Gln	6	9	ND	ND	ND	ND
Gly	0	1	0	0	124	93
Glu	0	0	15	0	64	86

FIG. 3. **Nickel ion binding to SlyD.** The number of bound nickel cations to SlyD was calculated from AES measurements (▲) and PPIase activity measurements (●). Each data point derived from two (for AES) or three (PPIase activity) independent measurements.

FIG. 4. **CD spectra of SlyD (*straight line*) and SlyD in the presence of 100 μM NiCl₂ (*dotted line*).** Spectra were recorded in 5 mM phosphate buffer, pH 6.5.

PAGE. For the substrate Suc-Ala-Phe-Pro-Phe-4NA, a specificity constant k_{cat}/K_m of 7,400 M^{-1} s^{-1} was determined. The substrate specificity in comparison with other PPIases from *E. coli* is summarized in Table I. The PPIase activity of SlpA was also measured with trypsin and the substrate Suc-Ala-Phe-Pro-Arg-4NA and was found to be 6,000 M^{-1} s^{-1}. FK506 up to 25 μM, CsA up to 100 μM, and NiCl₂ up to 100 μM did not decrease the PPIase activity of SlpA.

For interpretation of PPIase activity, the amino acid sequences of the N-terminal fragment of SlyD and SlpA were compared with the sequences of trigger factor and human FKBP12 (Fig. 1). The N-terminal fragment of SlyD shows only weak homology to both hFKBP12 (13.7% identity, 28.1% similarity) and trigger factor (15.1% and 27.4%, respectively). SlpA is 33.5% similar to hFKBP12 (18.1% identity) and 27.6% similar to trigger factor (12.8% identity).

Analysis of Nickel Binding to SlyD—From AES measurements, 3 bound nickel ions/SlyD molecule were observed (Fig. 3). The binding constants K_1, K_2, and K_3 for the three different binding sites were calculated to be 9.5×10^5 M^{-1}, 4.9×10^5 M^{-1} and 4.4×10^5 M^{-1}. The K_d value was calculated to 1.8 μM NiCl₂ (inflection point of the binding function).

CD spectra of SlyD were recorded without and with addition of 100 μM NiCl₂. The molar ellipticity spectra in the range from 190 to 260 nm are shown in Fig. 4. The maximum at 200 nm as well as the minimum at 215 nm were found to be not as intense in the presence of Ni^{2+} ions as without nickel ions. The shoulder around 228 nm remains almost unaffected. These changes suggest an increase in the amount of β-turn in the secondary structure (28).

DISCUSSION

PPIases were identified within all cell types and organisms, but little is known about their function *in vivo*. One commonly used strategy for investigation of protein function is the deletion or depletion of the appropriate gene followed by analysis of changes or defects of cellular functions. In *E. coli*, disruption of the gene encoding FKBP29 (fkbA) does not affect the phenotype (3). This may be due to functional redundancy of various proteins localized in the same cell compartment. This encouraged us to analyze two more proteins of *E. coli* with homology to FKBPs concerning their PPIase activity.

Chymotrypsin has been commonly used for isomer specific cleavage of the PPIase substrates in the protease coupled PPIase assay (1). Up to now, no proteolytic inactivation of PPIases was observed during the assay. This may be caused by inse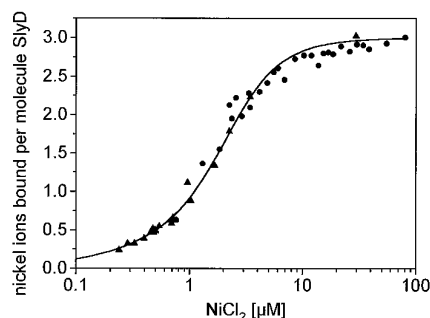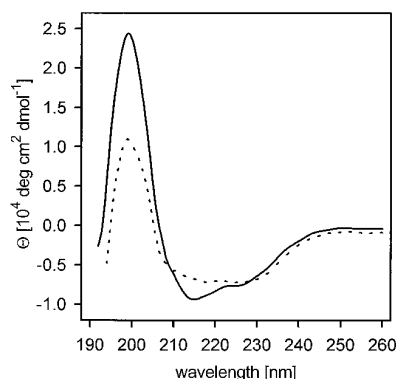nsitivity of most known PPIases to chymotrypsin. Another possibility is that the region responsible for PPIase activity remains intact if PPIases are proteolytically degraded during measurement (*e.g.* for *E. coli* trigger factor (29)).

For SlyD no PPIase activity was detected in the chymotrypsin-coupled assay. Therefore, an assay with trypsin, another isomer specific protease, was performed. On the basis of k_{cat}/K_m the PPIase activity of SlyD is considerably smaller than found for other PPIases with the same substrate (for Suc-Ala-Ala-Pro-Arg-4NA: SlyD 6, 200 M^{-1} s^{-1}, trigger factor of *E. coli* 499,000 M^{-1} s^{-1}). The protein fragment representing amino acids 1–146 of SlyD was purified without proteolytic degradation and expressed a quite similar k_{cat}/K_m compared with the full-length SlyD. This confirms that amino acids 1–146 are an independent structural region of SlyD. The C-terminal histidine-rich stretch of SlyD seems to be dispensable for PPIase activity. However, the PPIase activity of SlyD was inhibited by Ni^{2+} ions that probably bind to this region of the protein. Because of the reversibility of this inhibition, we do not attribute it to chemical modification. Since the activity of the isolated N-terminal fragment of SlyD was not affected, binding of nickel ions to SlyD may induce structural changes in the C-terminal domain, which in turn influence the PPIase activity of the N-terminal domain. This mechanism may be used *in vivo* to regulate the PPIase activity of SlyD. SlyD is the only PPIase known so far that is specifically controlled by high affinity metal ion binding. From CD spectra, alterations in the secondary structure of SlyD by nickel ion binding were deduced. These changes could be interpreted as an increase of the amount of β-turns. A turn-stabilizing effect of cations bound to the backbone amide or side chain functional groups has been occasionally observed for peptides (30).

The C-terminal domain of SlyD shows homology to histidine-rich regions of GTPases involved in generation of active hydro-

genases (HypB). Both the number of nickel ions bound per molecule SlyD (3) and the calculated K_d value for nickel cation binding to SlyD (1.8 μM) are in the same range as the values found in the literature for HypB. For HypB from *Bradyrhizobium japonicum* (24 clustered histidine residues), binding of 9 nickel ions with an K_d of 2.3 μM was detected (10); for HypB from *Rhizobium leguminosarum* (17 clustered histidine residues), 3.9 nickel ions/monomer with an K_d of 2.5 μM were detected (31). Hydrolysis of GTP by HypB has been found to be essential for nickel insertion in hydrogenases (32). Interestingly, HypB of *E. coli* lacks the histidine-rich region present at the N terminus of all other known HypB proteins and is not able to bind nickel but nevertheless exhibits GTPase activity (11). It is thus possible that SlyD plays the role of a nickel carrier in *E. coli* because its metal binding domain resembles those from HypB of other species. Thus SlyD could be involved in the generation of active hydrogenases in *E. coli*.

ORFs of *H. influenzae*, *P. fluorescens*, *E. coli* (ORF149), *E. aerogenes*, and *M. jannaschii* show high similarity to the N-terminal domain of SlyD (83.6, 54.9, 53.4, and 47.3%, respectively). After detection of PPIase activity for SlyD, similar activity was expected for the SlyD-homologous protein products of the ORFs mentioned. To confirm this, ORF149 of *E. coli* was cloned into an overexpression system. The purified protein SlpA showed a specificity constant k_{cat}/K_m of 7,400 $\mathrm{M}^{-1}\,\mathrm{s}^{-1}$ for the substrate Suc-Ala-Phe-Pro-Arg-4NA. The substrate specificity of SlpA shows a pattern different from other PPIases of *E. coli* (Table I). The closest similarity was found to the *E. coli* trigger factor PPIase except for higher activity with substrates containing isoleucine or tryptophan at P_1 position. By Western blotting with antibodies directed against recombinant SlpA, the protein product of ORF149 (SlpA) was now detected in *E. coli*. This was the first evidence for the expression of SlpA in *E. coli*.

The alignment of amino acid sequences of SlyD, SlpA, trigger factor, and hFKBP12 (Fig. 1) shows that SlyD and SlpA are distinct from FKBPs and trigger factor though a certain degree of homology exists. SlyD and SlpA are 28.1 and 33.5% similar to hFKBP12 and 27.4 and 27.6% to trigger factor, respectively. The similarity between hFKBP12 and trigger factor was calculated to 37.1%. From the alignment and the comparison of specificity constants, the SlyD-homologous proteins seem to be as distinct as trigger factor from FKBPs.

Of the amino acids highly conserved in FKBPs, only a few are conserved in SlyD and even less in SlpA (for review, see Ref. 33). Almost all FK506 binding residues are substituted in SlpA, except for Ala-82, Ile-92, and Phe-100. A Y27F mutation reduced the activity of hFKBP12 by 70% and binding of an FK506 homologue by 80% (34). This phenylalanine residue is present in SlpA. Of special interest is the amino acid substitution of Gly-29 in hFKBP12 to Val in SlyD and Leu in SlpA. These rather bulky residues are inserted into the possible binding pocket for FK506 (for hFKBP12, see Ref. 35). Moreover, both proteins as well as all other SlyD-homologues contain a large insertion before the last β-strand compared with the structure of FKBP (33). This could explain why the PPIase activity of SlyD and SlpA is not inhibited by FK506. Because of this insensitivity and the distance to hFKBP12 and trigger factor, but high sequence homology between the SlyD-homologous proteins, we presume these proteins to be members of a new subfamily of FKBPs.

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