Biochemical Properties of UspG, a Universal Stress Protein of *Escherichia coli*†

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ABSTRACT: Universal stress proteins (USPs) are abundant and widely distributed proteins. Even so, their mode of function is hardly understood. This study focuses on UspG (UP12) of *Escherichia coli*, which belongs to the UspFG subfamily. Resolution of UspG by two-dimensional gel electrophoresis uncovered a posttranslational modification during its overexpression in *E. coli*. One isoform represented the adenylated/phosphorylated state of UspG. In vitro experiments with His-tagged UspG revealed intrinsic autophosphorylation and autoadenylation activity. Moreover, covalently bound AMP could be released from UspG by piperidine treatment and subsequent thin-layer chromatography. UspG was characterized as a dimer, a property that got lost in a C-terminal truncated UspG. Moreover, the C-terminal part was found to be important for structural stability, because the truncation of six C-terminal amino acids resulted in a protein that was further truncated by 18 amino acids in vivo. The truncated UspG was still enzymatically active, albeit the activities were significantly reduced.

Proteins of the USP superfamily [Pfam PF00582 (*1*)] are characterized by a conserved protein domain consisting of 140−160 amino acids and are present in diverse organisms ranging from archaea and bacteria to yeast, fungi, and plants, encoded by multiple gene copies. Either one USP domain covers the whole USP protein or two USP domains are arranged on one polypeptide chain (tandem-type USP proteins). Some other proteins in bacteria, archaea, and plants contain one or two USP domains together with other functional domains (*2*). The six USP proteins of *Escherichia coli* can be divided into two subfamilies. UspA, UspC, and UspD belong to the UspA subfamily, whereas UspF and UspG are members of the second UspFG subfamily. UspE is a tandem-type USP protein whereby one domain is related to the UspA and the other to the UspFG subfamily (*3*). Interestingly, UspA from *E. coli* was found to be a serine and threonine phosphoprotein whose phosphorylation in vivo is dependent on the phosphotyrosine protein TypA (o591) (*4*, *5*).

In *E. coli*, four of the six USP proteins (UspA, UspC, UspD, and UspE) are coordinately regulated in a ppGpp-dependent manner under a large variety of conditions, such as stationary phase, starvation of carbon, nitrogen, phosphate, sulfate, and amino acids, increased temperature, oxidants, uncouplers, and other stimulants (*3*, *6*−*9*). A global transcriptional array approach uncovered elevated *uspG* (*ybdQ*) transcript levels in the early response to osmotic stress conditions (*10*) that might be regulated by the NtrB/NtrC two-component system in *E. coli* (*11*). Recent investigations using polyclonal antibodies against UspG revealed an increase of UspG levels at the stationary phase, under starvation conditions, in response to toxic agents (DNP,[1] CCCP) and increased temperature (44 °C) (*12*). The finding that UspG (from stationary phase) is released from the GroEL complex by the addition of ATP suggested that UspG might be a substrate for GroEL in *E. coli* (*12*).

The crystal structures of representatives from both subfamilies, the UspA protein from *Haemophilus influenzae* and the UspFG-type protein MJ0577 from *Methanococcus jannaschii*, revealed that both proteins form stable asymmetric homodimers involving the last six C-terminal amino acids (*13*, *14*). Although both structures show a quite similar fold, the two subfamilies exhibit different ligand specificities: MJ0577 (UspFG type) is probably a factor-dependent ATPase, showing a tightly bound ATP in its crystal structure, whereas UspA does not contain an ATP-binding pocket (*13*, *14*). These data are further strengthened by the fact that the *E. coli* UspF (YnaF, UP03) protein binds ATP (*15*).

Here, we report on posttranslational modifications of UspG, its biochemical properties, and the impact of the C-terminal domain on dimerization and protein stability. The role of posttranslational modification in regard of the regulatory mechanism of USP proteins is discussed.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions. The *E. coli* strains used in this work were BL21(DE3)/pLysS (*16*) and LMG194 (*17*). Cultures were grown aerobically in Erlenmeyer flasks in a rotary shaker at 37 °C. For liquid cultivation

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[1] Abbreviations: UspG(1−136)$_{tr}$, C-terminal truncated UspG lacking amino acids 137−142 due to genetic manipulation and amino acids 119−136 due to further proteolysis; CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DNP, α-dinitrophenol; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; PVDF, polyvinylidene fluoride; TLC, thin-layer chromatography.

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of *E. coli*, Luria−Bertani medium was used. When appropriate, the medium was supplemented with ampicillin (100 μg mL^{-1}) or chloramphenicol (34 mg mL^{-1}). Induction of His-tagged UspG production in *E. coli* LMG194/His$_6$-UspG-pBAD24 was carried out by addition of 0.006% arabinose.

Cloning of uspG and uspG(1−136). The *uspG* (*ybdQ*) gene (*18*) was amplified by PCR from purified *E. coli* MC4100 chromosomal DNA using the primers ybdQ-*Nde*I[2] sense, 5′-GGAGAAAGGCATATGTATAAGACAATC-3′, and ybdQ-*Bam*HI antisense, 5′-CTTATTCGGATCCTTAACGCACAACCAGCACCGG-3′. The PCR fragment obtained was digested with *Nde*I/*Bam*HI, gel purified, and ligated with appropriately digested pET-16b (Novagen). Cells of *E. coli* JM109 (*19*) were transformed with the ligation mixture. Afterward, *E. coli* BL21(DE3)/pLysS was transformed with the isolated and verified plasmid (pET-UspG) encoding the full-length UspG with an N-terminal His$_{10}$ tag separated by an amino acid linker consisting of nine amino acids (SSGHIEGRH).

Construction of His$_6$-UspG in pBAD24 was carried out using the primers UspG-*Nco*I-6H-sense, 5′-CATGCCATGGGCCATCATCATCATCATCATTATAAGACAATCATTATGCCAG-3′, and UspG-*Hin*dIII-antisense, 5′-GGGAAGCTTAACGCACAACCAGCACCGGCAGATT-3′. This construct was used for determination of modified UspG in whole cell extracts from the exponential and stationary phase of *E. coli* LMG194. This UspG derivative possesses only a His$_6$ tag without a linker of nine amino acids in comparison to the pET-UspG construct used for purification of His$_{10}$-UspG.

To obtain a C-terminal truncated His-tagged UspG(1−136)$_{tr}$, corresponding DNA fragments were obtained by PCR using pET-UspG as template DNA and the primers ybdQ-*Nde*I sense and ybdQ-(ΔC137−142)-*Bam*HI antisense, 5′-TGGCGGGATCCTTACAGATTGGCGTGGCGGATCACG-3′. Ligation of the truncated *uspG* gene with appropriately digested pET-16b resulted in the plasmid pET-UspG(1−136) whose integrity was confirmed by DNA sequencing.

Purification of UspG and UspG(1−136)$_{tr}$. The UspG and UspG(1−136)$_{tr}$ proteins containing a His$_{10}$ tag were purified from *E. coli* BL21(DE3)/pLysS/pET-UspG and BL21(DE3)/pLysS/pET-UspG(1−136), respectively. Cultures were grown aerobically in Luria−Bertani medium at 25 °C and induced with isopropyl thio-β-D-galactoside (IPTG, 0.5 mM) during exponential growth (OD$_{600}$ = 0.6) for 3 h (stationary phase). After centrifugation (15 min at 15000*g*, 4 °C), cells were washed at 4 °C in buffer W (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$). Subsequently, cells were resuspended and homogenized in buffer H [50 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 30 ng mL^{-1} DNase] and disrupted at 4 °C using a cell disruptor (IUL Instruments, Königswinter, Germany). Afterward, the obtained suspension was centrifuged (15 min at 15000*g*, 4 °C) followed by an ultracentrifugation step (1 h at 185000*g*, 4 °C) to separate soluble proteins from membranes and insoluble proteins.

The His-tagged UspG and UspG(1−136)$_{tr}$ were purified from the cytosolic fraction by affinity chromatography using nickel-NTA (triacetonitrile) agarose (Qiagen) equilibrated with buffer F [40 mM imidazole, 50 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 0.5 M NaCl, 10 mM 2-mercaptoethanol, 1.4 mM PMSF]. The His-tagged proteins were eluted with buffer F containing 250 mM imidazole and dialyzed three times against buffer D [50 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 80 mM NaCl, 1 mM 2-mercaptoethanol]. During the whole purification process the temperature was kept at 4 °C to minimize proteolysis. Detection of the purified His-tagged UspG was carried out by immunoblotting with monoclonal anti-mouse antibodies against the His tag (Qiagen) followed by incubation with alkaline phosphatase conjugated anti-mouse IgG (Rockland) in accordance to the manufacturer's instructions.

In Vitro Assay for Kinase Activity (Autophosphorylation). The assays were carried out according to Freestone et al. (*4*). A typical reaction mixture (25 μL) contained 1−3 μg of purified His$_{10}$-UspG or C-terminal-truncated His$_{10}$-UspG-(1−136)$_{tr}$ in buffer PA [20 mM Tris-HCl, pH 7.5, 10% (v/v) glycerol, 100 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 2 mM DTT] with the indicated concentrations of [γ-^{32}P]-ATP. The mixture was incubated at 37 °C, and at various time points the reaction was stopped by the addition of SDS loading buffer followed by separation of the proteins on a 12.5% SDS−polyacrylamide gel. Afterward, the gels were blotted onto nitrocellulose or PVDF membranes, which were exposed to a phosphoscreen for at least 24 h and subsequently scanned using a PhosphoimagerSI (Molecular Dynamics). Signal intensities of phosphorylated UspG were quantified using ImageQuant V5.0.

In Vitro Assay for Autoadenylation Activity. To test both UspG proteins for autoadenylation activity, the same reaction mixture and conditions were used as described for the kinase assay except for the use of [α-^{32}P]ATP as substrate.

Ligand specificity was tested using reaction mixtures (25 μL) containing 1 μg of purified His$_{10}$-UspG in buffer PA with either [α-^{32}P]ATP or [γ-^{32}P]ATP (specific radioactivity 99.3−1896 mCi/mmol) incubated at 37 °C for 1 h. Furthermore, unlabeled GTP or CTP was used in combination with radioactive ATP (3000 Ci/mmol) to test for reduction of radiolabeled UspG. The reactions were stopped by addition of SDS loading buffer, and proteins were separated on 12.5% SDS gels. Quantification of the amount of radiolabeled UspG was carried out as described above.

Piperidine Treatment of Adenylated His$_{10}$-UspG and Separation of AMP Using TLC. To demonstrate that AMP is bound to His$_{10}$-UspG when incubated with [α-^{32}P]ATP, radioactive protein bands were located and excised from the gel, sliced, and then eluted with 50 mM NH_4HCO_3/0.1% (w/v) SDS/1% (v/v) 2-mercaptoethanol by gentle shaking at 37 °C for 18 h. Eluates were cleared by centrifugation, and the samples were precipitated with 1.5 volumes of cold acetone using 50 μg of bovine serum albumin as carrier (*20*). After the precipitated His$_{10}$-UspG−AMP complex was split into two fractions, one sample was left untreated, whereas the other was treated with 0.5 M piperidine at 37 °C for 2 h. Piperidine was removed under vacuum (Speed-Vac). [^{32}P]-AMP released from adenylated His$_{10}$-UspG following piperidine treatment was dissolved in water and analyzed by thin-layer polyethyleneimine−cellulose F chromatography (Merck). TLC chromatography was done using 0.5% (v/v) formic acid as solvent followed by 0.15 M lithium formate, pH 3.0, as described (*21*). In parallel, internal standards (25

[2] Restriction sites are underlined.

nmol) of each AMP and ATP were separated and visualized with short-wave UV light for exact mapping of released [^{32}P]-AMP from UspG. The TLC plates were autoradiographed and visualized as described above.

Resolution of UspG on Two-Dimensional Polyacrylamide Gels (2D-PAGE). Isoelectric focusing (IEF) of whole cell extracts from LMG194/His$_6$-UspG-pBAD24 grown to exponential or late stationary phase containing comparable amounts of His$_6$-UspG as judged by 1D SDS gels (data not shown) was carried out using IPG strips with a nonlinear pH gradient between 3 and 10 (13 cm) on a IPGphor system (GE Healthcare). For assignment of the adenylated isoform of purified His$_{10}$-tagged UspG, 2.5 μg of protein preincubated in either the absence or presence of 10 μM (79.48 Ci/mmol) [α-^{32}P]ATP was used with IPG strips of the same pH gradient. The strips used were rehydrated for 19 h (20 °C, 50 μA, constant) in 250 μL of buffer R [8 M urea, 2 M thiourea, 2% (w/v) CHAPS, 1.25% (v/v) IPG buffer (pH 3−10), 28.4 mM DTT] and were focused for 19650 V h. Equilibration of the strips was carried out two times at room temperature (15 min) in buffer E [50 mM Tris-HCl, pH 6.8, 6 M urea, 30% (v/v) glycerol, 4% (w/v) SDS, 18.2 mM DTT] followed by second dimension analysis using SDS gels as indicated. The detection of UspG isoforms and the assignment of adenylated His-tagged UspG were accomplished by immunoblotting using anti-mouse antibodies against the His tag and autoradiography.

Stability Analysis of Phosphorylated Amino Acids. PVDF membranes containing the ^{32}P-labeled phosphoproteins were either heated to 43 °C in 1 M HCl for 90 min to test for acid stability or heated to 43 °C in 3 M NaOH for 90 min to test for alkaline stability. After treatment, the membranes were neutralized, dried, and reautoradiographed as described above.

Native Gel Electrophoresis. The separation of purified His$_{10}$-UspG and His$_{10}$-UspG(1−136)$_{tr}$ was performed according to Ornstein−Davis on 10% gels (*22*). As protein standards BSA (66 kDa), lysozyme (14.3 kDa), trypsin inhibitor (soybean, 20 kDa), and conalbumin (77 kDa) were loaded, and gels were visualized by silver staining (*23*).

Electrophoretic Mobility Shift Assays (EMSA). EMSA was performed by the method described (*24*). DNA fragments used were obtained in the following way: the primers ybdQ-inter sense, 5′-AACTCCGTCATCCTGGGCGA-3′, and ybdQ-inter antisense, 5′-AGGTCAGAGCCACAGATCGC-3′, were used to obtain the 217 bp intergenic *ybdQ*/*ybdR* region plus a 5′ 102 bp region of the *ybdQ* (*uspG*) gene and a 124 bp region of the adjacent *ybdR* gene. To test an influence on DNA topologies on the binding of His$_{10}$-UspG, two different synthetic DNA probes representing a curved DNA (60 bp SOA probe) and noncurved DNA (64 bp SOB probe) were used as decribed (*25*). Finally, pUC19 DNA cut with *Xma*I was UV cross-linked with His$_{10}$-UspG and separated on a 1% agarose gel.

Labeling of the DNA was carried out with 10 units of T4-polynucleotide kinase and 10 μCi of [γ-^{32}P]ATP in the appropiate buffer (50 mM Tris-HCl, pH 8.2, 10 mM $MgCl_2$, 0.1 mM EDTA, 5 mM DTT, 0.1 mM spermidine) at 37 °C for 1 h. Subsequently, the labeled DNA was purified using the nucleotide removal kit (Qiagen). DNA fragments with a radioactivity of 50000 counts were incubated with different concentrations of purified His$_{10}$-UspG in buffer [50 mM Tris-HCl, pH 7.5, 50 mM KCl, 2 mM $MgCl_2$, 10% glycerol (v/v), 1 mM DTT] with a final volume of 20 μL. Binding reactions were performed at room temperature for 10 min, and samples were run on a nondenaturing 5% polyacrylamide gel.

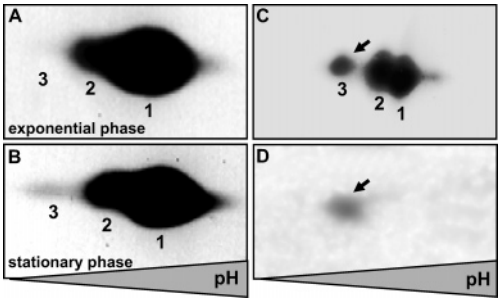

FIGURE 1: Isoforms of UspG and mapping of adenylated UspG by 2D-PAGE. Protein extracts with equal amounts of His$_6$-UspG protein from exponentially growing *E. coli* LMG194/His$_6$-UspG-pBAD24 (A) and from cells of the same strain at late stationary phase (B), purified His$_{10}$-UspG from *E. coli* BL21 (2.5 μg) (C), and purified His$_{10}$-UspG (2.5 μg) preincubated with 10 μM [α-^{32}P]-ATP (79.48 Ci/mmol) at 37 °C for 1 h (D) were separated by 2D-PAGE and detected by immunoblotting using anti-mouse antibodies directed against the His tag and, when appropriate, autoradiography. The numbers indicate the three UspG isoforms. The arrow indicates the adenylated form of His$_{10}$-UspG.

RESULTS

UspG Undergoes Posttranslational Modification during Its Overproduction in E. coli. Because UspG is a member of the UspFG subfamily, it seems reasonable to test whether this protein undergoes posttranslational modifications in vivo. Modifications by phosphorylation and/or adenylation would lead to changes in the isoelectric point (p*I*). As shown in Figure 1, 2D-PAGE analysis of cell extracts from *E. coli* LMG194 containing His$_6$-UspG indicated the formation of three different isoforms of UspG in the stationary phase (Figure 1B) whereas the third isoform is absent in exponentially growing cells (Figure 1A).

For in vitro investigations of the biochemical properties of UspG, we cloned the UspG encoding gene (*ybdQ*) into the expression vector pET-16b under control of a T7 promoter. Because we assumed that the C-terminal end of the USP proteins is involved in dimerization, for purification purposes, the UspG protein was equipped with an N-terminal His$_{10}$ tag separated by a linker comprising nine additional amino acids. The His$_{10}$-UspG protein (∼18.5 kDa) was overproduced and purified from the cytosolic fraction by nickel-affinity chromatography (Figure 2). The amount of soluble protein in the cytosolic fraction (Figure 2, lane 2) was sufficient to purify ∼0.5 mg of protein/L of culture to homogeneity as judged by silver-stained gels (Figure 2A, lane 3) and MALDI-TOF-MS (data not shown). Western blotting revealed that the monoclonal antibodies against the His tag recognized the purified His$_{10}$-UspG at ∼18.5 kDa together with a weak signal at ∼37 kDa (Figure 2B). The latter was found to correspond to the dimeric form of His$_{10}$-UspG (see below). The exact molecular mass of His$_{10}$-UspG was determined with 18315 Da (theoretical mass 18455 Da) by MALDI-TOF-MS analysis (data not shown). The difference between the theoretical and experimental protein mass indicated the cleavage of the initial formylmethionine from the protein during protein synthesis.

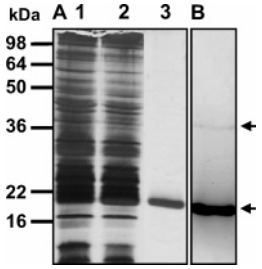

FIGURE 2: Purification of His_{10}-UspG. (A) Silver-stained 12.5% SDS gel of diverse cell fractions from *E. coli* BL21(DE3)/pLysS/pET-UspG. Lanes: 1, 20 μg of total cell extract (uninduced control); 2, 20 μg of the cytosolic fraction; 3, 2 μg of eluted protein after nickel-affinity chromatography. (B) Western blot with anti-His-tag antibodies. Immunoblotting of His_{10}-UspG (1.5 μg) was done as described in Experimental Procedures. Arrows indicate the positions of monomeric and dimeric His_{10}-UspG with apparent masses of ~18.5 and ~37 kDa, respectively.

Purified His_{10}-UspG from *E. coli* BL21pLysS/pET16-UspG (stationary phase) was resolved by 2D-PAGE, and again three isoforms were appeared (Figure 1C). The relative ratios of isoforms 1−3 were 2:1:0.1. Preincubation of purified His_{10}-UspG with [α-^{32}P]ATP allowed the identification of isoform 3 as the adenylated form of UspG (Figure 1D). The fraction of adenylated His_{10}-UspG after purification was determined to be 5% (Figure 1C). Besides an acidification of isoforms 2 and 3, both exhibited a slightly elevated molecular mass on 15% SDS gels. Their exact mass could not be determined because UspG (isoform 1) itself showed a broad mass peak in the linear mode in the mass spectrum including also apparent sinapinic acid products presumably overlapping with the modified UspG isomers (data not shown). Nevertheless, we can rule out that isoform 1 is a degradation product of the other two isoforms. No additional mass peak was detected using MALDI-TOF-MS corresponding to a mass significantly lower than that of isoform 1 (data not shown). Furthermore, based on in silico analysis a successive truncation of UspG from either the C- or N-terminus would always result in more acidic isoforms of UspG. The pretreatment of 10 μg of purified UspG protein with 10 units of calf intestinal alkaline phosphatase (CIP) resulted in a complete loss of isoform 3 after 2D gel electrophoresis, whereas this treatment was without effect on isoforms 1 and 2 (data not shown). Therefore, it can be ruled out that isoform 2 is phosphorylated. The modification of isoform 2 is unknown thus far, and its identification requires further experimentation.

Purified UspG Utilizes ATP as a Substrate for Its Autophosphorylation and Autoadenylation Activity. The incubation of purified His_{10}-UspG with [γ-^{32}P]ATP resulted in a fast time-dependent phosphorylation of the protein, indicating an intrinsic autophosphorylation activity (Figure 3A). Furthermore, incubation with [α-^{32}P]ATP resulted in a time-dependent labeling of UspG (Figure 3C). The enzymatic activities for UspG were determined to be 0.007 nmol/(min mg) for the phosphorylation and 0.003 nmol/(min mg) for the adenylation reaction (Figure 3B, D). As a control, purified His_{10}-UspG was denatured with 2% SDS before the addition of radiolabeled [γ-^{32}P]ATP or [α-^{32}P]ATP substrates, and no significant labeling of the protein was detectable (data not shown). Moreover, addition of 2% SDS after radiolabeling of the protein was without significant loss of the signal intensity, indicating a covalent binding of phosphate or AMP to UspG (data not shown). For direct proof of a covalently bound AMP moiety to UspG, [α-^{32}P]ATP-labeled UspG was treated with piperidine. As shown in Figure 4, [^{32}P]AMP was released from the ^{32}P-labeled UspG after treatment with 0.5 M piperidine. These results indicate that UspG possesses both intrinsic autophosphorylation and autoadenylation activity. Both modifications were acid-resistant but alkali-labile, being consistent with a phosphorylation and adenylation at serine or threonine residues (Figure 5). The search for modified peptides by tryptic digestion of purified His_{10}-UspG using peptide mass fingerprinting (PMF) was unsuccessful thus far although we have applied various methods including MALDI-TOF-MS in the linear and reflector mode, ESI (−) TOF MS, and ESI (−) precursor ion scan analyses (data not shown).

Nucleotide Specificity of UspG. When a constant amount of either [γ-^{32}P]ATP or [α-^{32}P]ATP (3000 Ci/mmol each) was used, a concomitant decrease of the radiolabeling degree of UspG with an increase of the amount of unlabeled ATP was observed. For example, the presence of 2 mM unlabeled ATP (labeled:unlabeled ATP ratio 1:30000) decreased the amount of radioactive phosphorylated or adenylated UspG to 10−15% (Figure 6). Increasing concentrations of the nucleotide GTP also significantly lowered the radiolabeling degree of UspG when incubated with [γ-^{32}P]ATP or [α-^{32}P]-ATP (Figure 6). In contrast, the tested pyrimidine nucleotide CTP was without significant effect. These results suggested a discrimination of UspG between purine and pyrimidine nucleotides. Presumably GTP competes with ATP for the nucleotide-binding pocket in UspG, whereas CTP does not.

UspG Forms Stable Homodimers via Its C-Terminal Domain. The in silico model predicted a dimeric structure of UspG via its C-terminal ends (Figure 10A, arrows). On the basis of this assumption, we experimentally characterized the oligomerization state of UspG from *E. coli*. To test the impact of the C-terminal domain on the dimerization, a truncated UspG lacking the last six C-terminal amino acids was synthesized. Surprisingly, after purification the truncated UspG migrated in SDS gels at a lower molecular mass than predicted by the amino acid sequence (Figure 7). By means of MALDI-TOF-MS spectrometry the relative molecular mass of the truncated UspG was determined to be 15782 Da, indicating a loss of an additional 18 amino acids from the C-terminus because His_{10}-UspG$(1-136)_{tr}$ was still detectable by Western blotting with the monoclonal antibodies against the N-terminal His tag (Figure 7A). Therefore, this UspG derivative was termed His_{10}-UspG$(1-136)_{tr}$. To test the oligomeric state, both wild-type UspG and its C-terminally truncated derivative were run on native polyacrylamide gels (Figure 8). Whereas wild-type UspG was detected in a dimeric form, the truncated derivative appeared only as a monomeric form.

The C-Terminal Part Is Important for Optimal Intrinsic Activities of UspG$(1-136)_{tr}$. To answer the question whether the C-terminal part (dimer interface) in UspG is of importance for its intrinsic enzymatic activities, we analyzed whether UspG$(1-136)_{tr}$ was still enzymatically active. For this purpose, purified His_{10}-UspG$(1-136)_{tr}$ was incubated with either [α-^{32}P]ATP or [γ-^{32}P]ATP. For each substrate a radiolabeling of the UspG$(1-136)_{tr}$ was detectable, though the labeling degree was reduced by about 80% compared to

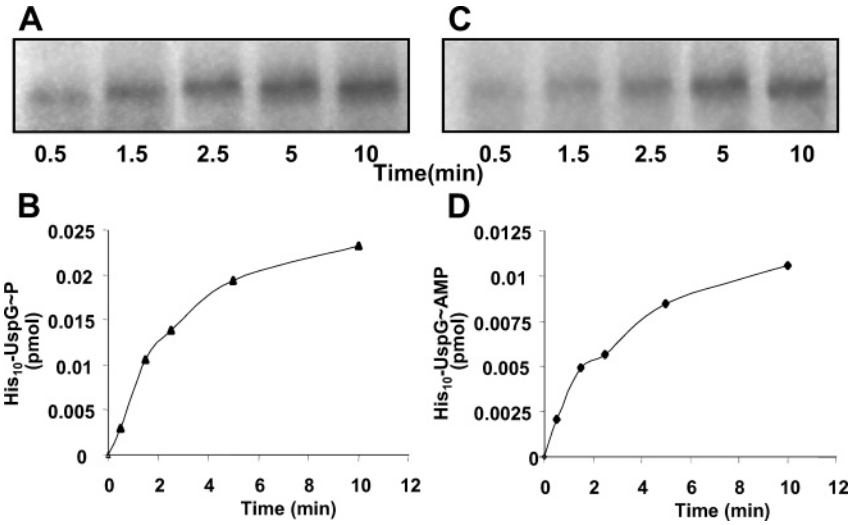

FIGURE 3: Time-dependent modification of His_{10}-UspG in vitro. (A) Autophosphorylation of UspG (1 μg per lane) after incubation with 1 mM [γ-^{32}P]ATP (198.6 mCi/mmol) as substrate. (C) Autoadenylation of UspG (1 μg per lane) using 1 mM [α-^{32}P]ATP (198.6 mCi/mmol). At the indicated time points, the reaction was stopped, and proteins were analyzed by 12.5% SDS gel electrophoresis followed by autoradiography of the blotted gels to nitrocellulose membranes. Quantification of the amount of phosphorylated (B) and adenylated (D) UspG was determined using ImageQuant.

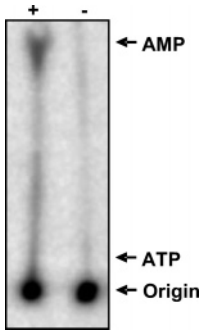

FIGURE 4: Release of [^{32}P]AMP from [α-^{32}P]ATP-incubated His_{10}-UspG using piperidine. Purified His_{10}-UspG (3 μg) was incubated with 10 μM [α-^{32}P]ATP (79.48 Ci/mmol) at 37 °C for 1 h, gel-purified, precipitated, and treated with (+) and without (−) piperidine as described in Experimental Procedures. Resolution of the piperidine hydrolysis products was carried out by TLC using AMP and ATP as internal standards (25 nmol each). Nucleotides were visualized in short-wave UV light.

FIGURE 5: pH-stability profile of radiolabeled UspG. Purified His_{10}-UspG (1 μg) was incubated with 10 μM (A) [α-^{32}P]ATP or (B) [γ-^{32}P]ATP (19.86 Ci/mmol) as described in Experimental Procedures and separated by SDS gel electrophoresis. The proteins were transferred to a PVDF membrane and incubated with either 1 M HCl at 43 °C for 1.5 h to test for acid stability or 3 M NaOH at 43 °C for 3 h to test for alkaline stability. Lanes: 1, reference signal of adenylated UspG incubated in 100 mM Tris-HCl, pH 7.5; 2, acid treatment of adenylated UspG; 3, alkaline treatment of adenylated UspG; 4, reference signal of phosphorylated UspG in 100 mM Tris-HCl, pH 7.5; 5, acid treatment of phosphorylated UspG; 6, alkaline treatment of phosphorylated UspG.

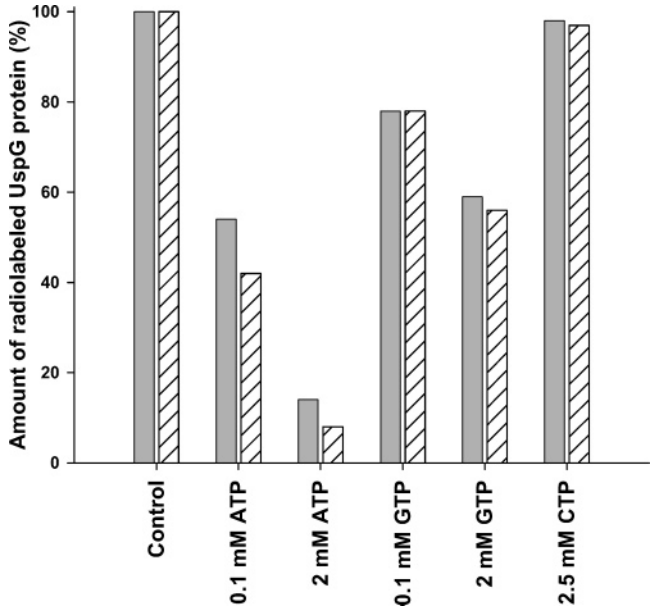

FIGURE 6: Substrate specificity of His_{10}-UspG. The figure represents the influence of ATP, GTP, and CTP on the percentage of radiolabeled UspG. Purified His_{10}-UspG (1 μg) was incubated with 5 μCi of [α-^{32}P]ATP (gray) or [γ-^{32}P]ATP (hatched) (3000 Ci/mmol each) together with the indicated concentration of either unlabeled ATP, GTP, or CTP at 37 °C for 1 h. The reaction was stopped by adding SDS loading buffer. Separation of proteins was done by SDS−PAGE. Quantification of the radiolabeled UspG was performed using ImageQuant. For comparison, the amount of labeled UspG without the addition of cold nucleotides was set to 100% (control).

the wild-type protein (Figure 9). However, the labeling was specific, because (i) an excess of unlabeled ATP reduced the amount of the radioactively labeled protein significantly (Figure 9, lanes 3) and (ii) the denatured protein exhibited no enzymatic activities (data not shown).

Electrophoretic Mobility Shift Assays. Because of sequence homologies of the N-terminal region of USP proteins to eukaryotic transcriptional regulators [MADS-Box protein family (*26*, *27*)], it was assumed that USP proteins might bind to DNA (*28*). Since this was never tested experimentially, we incubated purified His_{10}-UspG protein with distinct DNA fragments such as (i) the intergenic *ybdQ/ybdR* DNA region, (ii) cut pUC19 DNA, and (iii) synthetic DNA probes applied for DNA-binding proteins which prefer rather

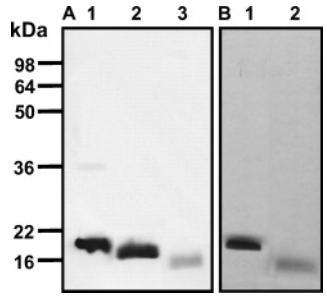

FIGURE 7: Purification and stability of His_{10}-UspG(1−136)$_{tr}$. (A) Western blot with anti-His-tag antibodies. Lanes: 1, 1.5 μg of purified His_{10}-UspG; 2, 25 μg of whole cell extracts from *E. coli* BL21(DE3)pLysS/pET-UspG(1−136) before and after purification (lane 3, 0.5 μg). (B) Silver-stained 12.5% SDS gel of purified His_{10}-UspG (lane 1, 1.5 μg) and His_{10}-UspG(1−136)$_{tr}$ (lane 2, 0.5 μg).

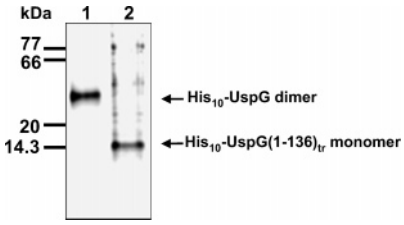

FIGURE 8: Native gel electrophoresis of UspG and UspG(1−136)$_{tr}$. Purified His_{10}-UspG (3 μg) (lane 1) and His_{10}-UspG(1−136)$_{tr}$ (2 μg) lacking the C-terminal dimerization domain (lane 2) were separated on a 10% native gel and visualized by silver staining. Native protein standards used: lysozyme (14.3 kDa), trypsin inhibitor (soybean, 20 kDa), BSA (66 kDa), and conalbumin (77 kDa).

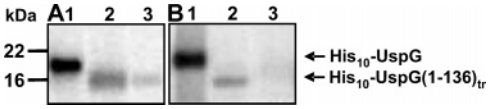

FIGURE 9: In vitro adenylation and phosphorylation of His_{10}-UspG und His_{10}-UspG(1−136)$_{tr}$. The figure represents the autoradiogram of purified His_{10}-UspG und His_{10}-UspG(1−136)$_{tr}$ (each 1 μg) labeled with 10 μM [α-^{32}P]ATP (A) or [γ-^{32}P]ATP (B) (19.86 Ci/mmol each) at 37 °C for 30 min after transfer to PVDF membranes. Lanes: A1 and B1, His_{10}-UspG; A2 and B2, His_{10}-UspG(1−136)$_{tr}$; A3 and B3, His_{10}-UspG(1−136)$_{tr}$ with 1 mM ATP (198.6 mCi/mmol).

specific DNA topologies than specific DNA sequences (*25*). Although the amount of His_{10}-UspG protein used was varied over a wide range (protein:DNA ratio of 1:1 to 1000:1), we were unable to detect any specific protein−DNA complex irrespective of the DNA used (data not shown).

DISCUSSION

In nature, posttranslational modification (e.g., phosphorylation, nucleotidylation) of proteins is a widespread mechanism employed to alter enzymatic activities of proteins (*29*, *30*). In the case of the universal stress proteins, posttranslational modification has only been shown for UspA of *E. coli*, which is phosphorylated by the phosphotyrosine protein TypA at yet unknown serine and threonine residues (*4*, *5*). Furthermore, overexpression of UspA led to changes in the isoelectric points of at least six other proteins (*31*).

In this report, application of a 2D gel electrophoresis approach identified three isoforms of His_6-UspG within whole *E. coli* LMG194 cell extracts from stationary phase cells or purified His_{10}-UspG from another *E. coli* BL21 strain. These isoforms are characterized by two different acidification states in the first dimension and a slightly higher molecular mass in the second dimension compared to the unmodified isoform 1. Notably, the fraction of isoform 3 of His_{10}-UspG detected after purification of UspG isoforms was even more distinctive in the BL21 strain during stationary phase compared to the whole cell extracts of the LMG194 strain before the purification procedure. This discrepancy might be related to the different expression systems and/or the *E. coli* strains used. Furthermore, we cannot rule out that isoform 3 of UspG was selectively enriched during the purification procedure.

Because of the harsh denaturing conditions during 2D-PAGE, we concluded that the isoforms were the results of covalent modification(s) of UspG. In vitro experiments with purified His_{10}-UspG confirmed posttranslational modifications by intrinsic autophosphorylation and autoadenylation activities. Furthermore, the treatment of radioactive labeled His_{10}-UspG with piperidine resulted in a release of [^{32}P]AMP from adenylated UspG. The herein detected acid stability of both modifications indicated a phosphorylation and/or adenylation at serine or threonine residues.

There are only some other examples known for posttranslational modifications in form of an adenylation. A classical example is the glutamine synthetase (GlnA) of *E. coli*. This protein is reversibly adenylated by a second protein (adenyltransferase) on a highly conserved tyrosine residue, leading to a reduced activity of GlnA (*35*, *36*). For the large tumor antigen protein (SV40-T), which is important for viral DNA replication in simian virus 40, adenylation of a serine residue has been described as well as for the ICP4 protein, the major regulator of the herpes simplex virus which undergoes adenylation and guanylation (*20*, *37*, *38*). Finally, the MAFP protein [major acidic fibroblast growth factor (aFGF) stimulated phosphoprotein], a glycoprotein from bovine liver, shows both autophosphorylation and autoadenylation activity which occurs at the same threonine residue and is stimulated by aFGF (*39*).

Isoform 3 of UspG on the 2D gels could be assigned to the adenylated form of UspG, which was only detectable in cells of the stationary growth phase. It represents approximately 5% of the loaded UspG protein. In vivo, it is hard to imagine that only a small fraction of UspG is modified. The partial adenylation of UspG observed in vitro might be related to a short half-time of the modification, as was observed for CheY−P (*32*), or an underestimation of the modification due to in vitro manipulations, as in case of the sensor kinase KdpD (*33*). Interestingly, treatment of purified UspG with alkaline phosphatase resulted in the disappearance of isoform 3 after 2D gel electrophoresis, whereas this treatment was without effect on isoforms 1 and 2. This result indicates that isoform 2 is not the phosphorylated isoform. It seems as though isoform 3 represents the phosphorylated and adenylated form. Although various techniques were applied, PMF analysis of phosphorylated/adenylated UspG (isoform 3) was yet unsuccessful in identifying the modification sites. PMF approaches might fail due to the instability of the modification(s) during MALDI analyses. The nature of the modification of the second isoform of UspG still needs to be explored. In analogy to UspA, whose in vivo phosphorylation is dependent on the ribosomal tyrosine phosphoprotein TypA (*5*), a yet

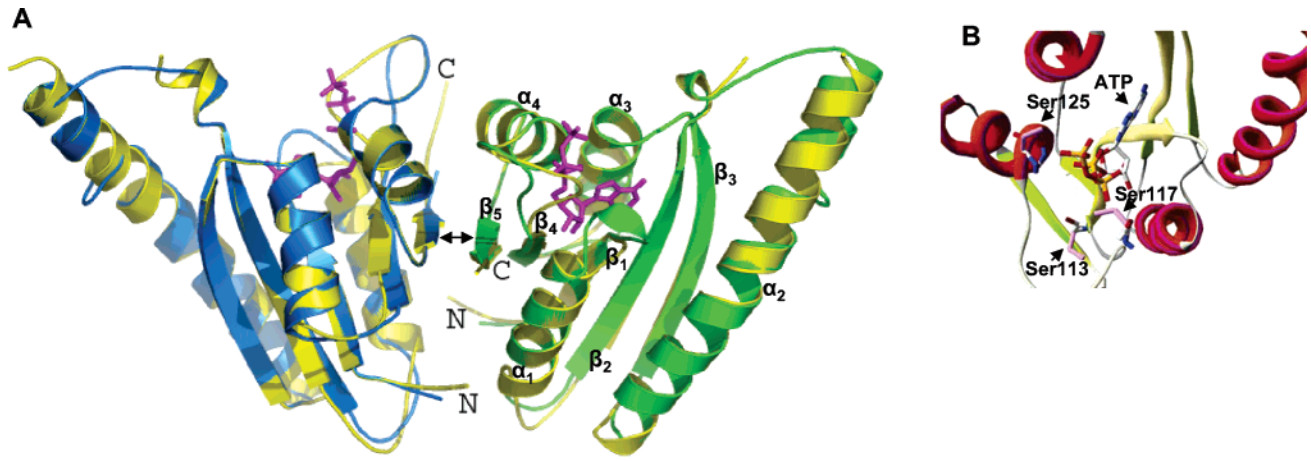

FIGURE 10: (A) Tertiary structure of MJ0577 (*M. jannaschii*) and the modeled 3D structure of UspG (*E. coli*). The dimeric model of UspG is shown with bound ATP (pink) as in MJ0577. The blue and green ribbons represent individual monomers of the UspG dimer. The yellow ribbons represent the dimeric structure of MJ0577. The arrows indicate the C-terminal-located dimer interface. (B) 3D model of the ATP-binding pocket of UspG. The side chains of the potential involved amino acid residues for modification (Ser113, Ser117, Ser125) are indicated in magenta. The models were generated using Pymol [DeLano Scientific LLC, San Carlos, CA (http://www.pymol.org)].

unknown factor might also stimulate the modification of UspG in vivo during the stationary phase where UspG has been shown to be increasingly synthesized (*12*). Nevertheless, whether UspG is also posttranslationally modified during other stress conditions remains unclear thus far and needs to be tested.

To identify potential modification sites within UspG, the 3D structure of UspG was modeled with bound ATP. As shown in Figure 10B, three potential serine residues (Ser113, Ser117, Ser125) are proposed, which are located in near proximity to the ATP-binding pocket (*34*). These amino acid residues are hot candidates for single amino acid replacement studies.

It has been published that synthesis of UspG is stimulated by heat shock (*12*), and we found a 3-fold induction by a 4% ethanol stress 1 h after addition of ethanol at OD_{600} = 0.5 using *E. coli* MG1655 in phosphate-buffered minimal medium supplemented with 0.4% (w/v) glucose (*10*) and 10 mM K^+ (this work; data not shown), both conditions triggering the heat shock response in *E. coli* (*40*). Bibi and co-workers have demonstrated that, by adding ATP, UspG is released from the heat shock protein GroEL, which belongs to the highly conserved Hsp60 family of chaperones (*41*). GroEL plays, together with GroES, an important role in protein folding, and it is assumed that UspG requires GroEL for a proper conformation (*12*). Considering that UspG itself uses ATP as a substrate as demonstrated in this work, it needs to be explored whether UspG is released from GroEL by ATP hydrolysis solely mediated by GroEL or is released as a functional component under some stress conditions (e.g., stationary phase) from the GroEL complex by modification of UspG. Such cooperation might be important for UspG to perform its role during recovery of *E. coli* from the stationary phase, a phenotype which has been reported for a *uspG* deletion strain (*12*).

Structural data for the UspG homologous proteins MJ0577 from *M. jannaschii* (*14*), the UspA protein from *H. influenzae* (*13*), the hypothetical USP protein Rv1636 from *Mycobacterium tuberculosis* H37Rv (protein database: http://www.rcsb.org/pdb/; PDB code 1TQ8), and the putative universal stress protein AQ_178 from the hyperthermophilic bacterium *Aquifex aeolicus* (PDB code 1Q77) revealed the C-terminal part as a dimer interface. Remarkably, truncation of six C-terminal amino acids in UspG not only prevented dimer formation but also increased protease accessibility, resulting in a further C-terminal truncation of the protein by 18 amino acid residues. Nevertheless, this truncated protein still exhibited autophosphorylation and autoadenylation activities, albeit the in vitro values were significantly reduced (20% in comparison to wild type). Therefore, it can be concluded that the C-terminal part of UspG is of structural importance and necessary for the formation of UspG dimers. Keeping in mind that His_{10}-UspG runs exclusively in its dimeric state during native gel electrophoresis, it seems plausible that dimer formation is a prerequisite for its optimal intrinsic enzymatic activities. Despite the truncation, residual enzymatic activities are conceivable, because most of the putative amino acid residues involved in binding of ATP and phosphorylation are still present in His_{10}-UspG$(1-136)_{tr}$. Particularly, Asp13, Val41, Gly112, and Ser113 (*14*) might be responsible for binding of the adenine and ribose moieties of ATP. Furthermore, the truncated His_{10}-UspG$(1-136)_{tr}$ still carries two of the potential modification sites (Ser113 and Ser117, Figure 10B).

Searches for similar protein folds revealed 28% sequence identity of UspG with the USP protein MJ0577 of *M. jannaschii*, an ATP-binding protein of still unknown function. Due to sufficient sequence identity of UspG compared to the template protein from MJ0577 (PDB code 1MJH), we were able to build an in silico model of a UspG dimer (Figure 10A) employing the fully automated comparative SWISS-MODEL protein modeling server (*42*, *43*). This model indicates the conservation of the dimer structure using a conserved C-terminal part (β_5 sheet) as observed in the crystal of MJ0577 and the typical α/β structure consisting of five β-sheets arranged in a characteristically 3-2-1-4-5 parallel order surrounded by two α-helices located on each side of the sheets. A similar fold was observed in the other USP proteins, indicating that it represents a common structural motif in the USP family (*13*−*15*).

On the basis of the observations reported here and those reported for UspA (*4*, *31*), we propose that posttranslational modification of USP proteins is the key feature of the regulatory mechanism of this protein class. At least for UspG,

it was shown that this protein lacks DNA-binding properties, suggesting that USP proteins are unable to regulate gene expression directly. Rather, it is conceivable that the phosphorylated/adenylated forms of USP proteins interfere with signaling events such as the scaffolding proteins of eukaryotes (*44*) or mark proteins by tagging or support proper folding and/or GroEL activation as in case of UspG (*12*). A recent accomplished large-scale analysis of protein complexes in *E. coli* has reported at least 14 potential candidates that interact with UspG. Among them are DnaJ, DnaK, the small heat shock protein IbpA, proteins involved in active protein export (SecA, SecB), 50S ribosomal proteins (RplL, RplW), EF-Tu, enzymes for lipopolysaccharide (HldD) and pyrimidine (PyrH) biosynthesis, regulators such as MreB, and some hypothetical proteins (YcaO, YacL) (*45*). Finding the targets of modified UspG will be the next goal to strengthen this hypothesis further.

In this report we focused on the biochemical characterization of UspG, but in the future it will be of high interest to investigate the impact of chemically modified UspG protein on the proteome of *E. coli* at different growth phases and under other stress conditions (e.g., CCCP or ethanol) in comparison to *uspG* mutants that are insensitive to modification.

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