Cascade Control of Escherichia coli Glutamine Synthetase

PROPERTIES OF THE PII REGULATORY PROTEIN AND THE URIDYLYLTRANSFERASE-URIDYLYL-REMOVING ENZYME

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SUMMARY

The PII regulatory protein of Escherichia coli glutamine synthetase exists in two interconvertible forms: a uridylylated form (PIID) which promotes the deadenylylation of glutamine synthetase and an unmodified form (PIIA) which promotes the adenylylation of glutamine synthetase (Mangum, J. H., Magni, G., and Stadtman, E. R. (1973) Arch. Biochem. Biophys. 158, 514–525). PIIA has been purified to homogeneity. Its molecular weight is 44,000. The protein is composed of four subunits, each with a molecular weight of approximately 11,000. The subunits are identical as judged by: (a) the homogeneity of the subunits in sodium dodecyl sulfate, 8 M urea, and 6 M guanidine HCl; (b) the minimal molecular weight calculated from the amino acid composition; and (c) the isolation of only two tryptic peptides containing tyrosine (there are 8 tyrosyl residues per 44,000 molecular species). Following iodination of PIIA and PIID with 125I in the presence of chloramine-T, tryptic digestion yields two radioactive peptides from PIIA and only one from PIID. Since a tyrosine with a substituted hydroxyl group cannot be iodinated, this result indicates that 1 tyrosyl residue in each subunit is modified by the covalent attachment of UMP. This conclusion is supported also by the fact that treatment of PIID with snake venom phosphodiesterase results in the release of covalently bound UMP and the stoichiometric appearance of phenolate ion (pH 13) as measured by ultraviolet absorption spectroscopy.

The enzyme activities (uridylyl-removing) responsible for removal and (uridylyltransferase) responsible for attachment of UMP to PII have been partially purified. These activities co-purify through a variety of procedures, including hydrophobic chromatography, and are stabilized by high ionic strength buffers. Whereas Mn2+ alone supports only uridylyl-removing activity, ATP, a-ketoglutarate, and Mg2+ support both uridylyl-removing and uridylyltransferase activities.

ATTASE ATP + GS → AMP-GS + PPi

AMP-GS + P i → AMP-GS + ADP

SCHEME

Recent work from this laboratory has demonstrated that the regulatory protein PII exists in two interconvertible forms (7). As is shown in Scheme 1, one form, PIIA, stimulates the adenylyltransferase-catalyzed adenylylation of glutamine synthetase, whereas the other form, PIID, is required for the adenylyltransferase-catalyzed deadenylylation. When PIIA is incubated in the presence of UTP, ATP, a-ketoglutarate (aKG), Mn2+ or Mg2+, and another enzyme, uridylyltransferase (UTase), it is converted to PIID. This conversion involves the covalent attachment of UMP to the protein (Reaction 1) (8).

UTP + P ii → UTase aKG, ATP, Mg2+ → P ii, UMP + PPi

REACTION 1

P ii can be regenerated from PIID by a uridylyl-removing enzyme activity (uridylyl-removing enzyme) (Reaction 2).

PIID + UMP → UR enzyme Mn2+ → P ii + UMP

REACTION 2

1 The abbreviations used are: ATase, adenylyltransferase; GS, glutamine synthetase; aKG, a-ketoglutarate; UTase, uridylyltransferase; UR, uridylyl-removing.

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In addition to its control by cumulative feedback inhibition and repression, the glutamine synthetase activity of Escherichia coli is regulated by the covalent attachment and removal of AMP from a specific tyrosyl residue in each of the enzyme's 12 identical subunits (1–4). Adenylation of a subunit converts it to a less active form dependent upon Mn2+ (1). The enzyme's activity is thus controlled by the average number of adenylylated subunits per molecule which can vary from 0 to 12. Both adenylylation and deadenylylation of glutamine synthetase are catalyzed by a single enzyme, adenylyltransferase (ATase)1 (5). Adenylylation involves a transfer of the AMP moiety of ATP into an AMP-0-tyrosyl linkage (4), whereas deadenylylation involves a phosphorolysis of the latter to yield ADP (5). Although adenylyltransferase catalyzes both reactions, its ability to adenylylate or deadenylylate glutamine synthetase (GS) is modulated by the regulatory protein PII and metabolic effectors including a-ketoglutarate, ATP, glutamine, and inorganic phosphate (Pi).
We now report the purification of PII to homogeneity, demonstrate that the protein is a tetramer of identical subunits, and that, like glutamine synthetase, its activity is modulated by the covalent attachment of a nucleotide to a specific tyrosyl residue in each of the subunits. In addition, the protein(s) exhibiting UR and uridylyltransferase activities has been partially purified and characterized, and evidence is presented suggesting that both activities may be a property of the same enzyme or enzyme complex. Preliminary reports of this work have appeared (9, 10).

EXPERIMENTAL PROCEDURES

[U-3H]UMP and [3H]uridylyltransferase activity were measured by incubating 77.4 units of PIIA with 125I were obtained from New England Nuclear Corp. Unlabeled nucleotides were obtained from P-L Biochemicals, a-ketoglutarate from Calbiochem, DEAE-cellulose (DE52) from Whatman, and agarose (Bio-Gel A-0.5m, 100 to 200 mesh) and hydroxyapatite from Bio-Rad. Sepharose 4B-(CH2)6-NH2 was prepared by the method of Sissittle (11, 12) or obtained commercially from Pharmacia (Al-Sepharose 4B). All other chemicals used were reagent grade.

Glutamine synthetase was purified from Escherichia coli W cells by a zince precipitation method (13). The preparation of 5'-[14C]-adenylated glutamine synthetase used as substrate in the deadenylation assay is described elsewhere (14).

Cell Growth—E. coli W (ATCC 9637) cells were grown on 50 mL NH4Cl-0.66 g glyceral-buffered salts medium in a 400-liter fermentor. Cells were harvested approximately 3 hours after the onset of stationary phase. The cell paste was frozen in liquid nitrogen and stored at -20°C.

Enzyme Assays—Glutamine synthetase was assayed by the γ-glutamyl transferase assay (15). One unit of activity is equal to a 1.0 μmol of γ-glutamyl hydroxamate formed per min at 37°C.

Adenylyltransferase was assayed at 37°C in a 100-μL reaction mix containing 100 mM ATP, 20 mM Tris-HCl, pH 7.8, 20 mM MgCl2, 10 mM glutamine, 0.0 μmol of unadenylated glutamine synthetase, and enzyme. Aliquots were withdrawn after various periods of incubation for the determination of the adenylylated γ-glutamyl transferase activity in the presence of Mn2+ and Mg2+, where only unadenylated subunits are active (15). Adenylyltransferase adenylylation activity is thus reflected by a decrease in the γ-glutamyl transferase capacity of glutamine synthetase. One unit of activity is arbitrarily defined as that amount of adenylyltransferase necessary to decrease the glutamine synthetase-catalyzed formation of γ-glutamyl hydroxamate in the presence of Mn2+ and Mg2+ by 1.0 μmol (ΔA280 = 0.30) per min.

Pm1 activity was measured as previously described (7). Twenty-seven units of adenylyltransferase (measured by the method of Ginsburg (14)) were used per 0.1 mL of assay mix. One unit of adenylylation activity is that amount of Pm1 required to remove 1 pmol of [3H]AMP from glutamine synthetase per min at 37°C.

Uridylyltransferase activity was measured by incubating 77.4 units of PIIA (a mixture of PIIA and Pm1 which was at least 95% PIIA) and enzyme at 37°C in 50-μL reaction mixtures containing: 50 mM 2-mercaptoethanol (pH 7.4), 100 mM KCl, 10 mM MgCl2, 0.1 mM ATP, 0.2 mM UTP, 5 mM a-ketoglutarate, and 1 mM diethylthreitol. After a suitable period of time at 37°C an aliquot was withdrawn and the increase in Pm1 activity measured. Under these conditions uridylyltransferase activity is linear for at least 2 hours and proportional to enzyme concentration. The Pm1 used as substrate was purified through Step 4 (agarose chromatography) of the Pm1 purification procedure (see "Results").

Uridylyl-removing activity was assayed by either of two methods: (1) Decrease in Pm1 activity was followed by incubation at 37°C of 130 units of Pm1 in a 30-μL reaction mixture containing 50 μM 2,4-dimethylimidazole (pH 8.6), 100 mM KCl, 1 mM MnCl2, and enzyme. After 30 min 70 μl of deadenylation reaction mixture were added and the residual Pm1 activity measured. Activity is expressed as the percentage of Pm1 activity inactivated. (2) Release of [3H]UMP ([400 pmol/ml] from PIIA was followed by incubating 0.08 units of PIIA containing covalently bound [3H]UMP in a reaction mixture as described above (Method 1). After 30 min 4 μl of bovine serum albumin (30 mg/ml) and 150 μl of perchloric acid (7%) were added to terminate the reaction. The mixtures were centrifuged and 150 μl of the supernatant were counted in Aquasol (New England Nuclear Corp.). Activity is expressed as picomoles of UMP released per μl of enzyme.

Uridylyltransferase-catalyzed formation of γ-glutamyl hydroxamate in the presence of Mn2+ and Mg2+.

[3H]UMP-Pm1 (PIID) was prepared by incubating PIIA (a mixture of PIIA and Pm1 that was at least 95% PIIA) in the presence of [3H]UTP and uridylyltransferase under the conditions described for assaying uridylyltransferase activity. The reaction was allowed to proceed until there was no further increase in Pm1 activity, then 20 mM Na EDTA was added to stop the reaction and the mixture was dialyzed exhaustively against the standard buffer containing 20 mM 2-mercaptoethanol (pH 7.6), 10 mM 2-mercaptoethanol, and 0.1 mM K2Mg EDTA, and the Pm1 was purified by hydroxyapatite chromatography (see "Results").

Protein concentration was determined either by the method of Lowry (16) or, for protein solutions greater than 10 mg/ml, by the biuret method (17).

Gel Electrophoresis—Polyacrylamide gel electrophoresis was carried out in tubes (0.5 × 8 cm) at pH 9.5, 25°C, according to the procedures of Ornstein (18) and Davis (19). Electrophoresis in acrylamide gels containing 0.1% sodium dodecyl sulfate was carried out at 25°C according to the procedure of Weber and Osborn (20). Staining with Coomassie blue and destaining was done by standard methods (20). Electrophoresis in gels containing 8 M urea was carried out at 25°C as described by Reisfeld and Small (21). Protein samples used in these experiments were heated for 10 min at 65° in the presence of 8 M urea and 10 mM dithiothreitol prior to electrophoresis.

Sedimentation Equilibrium—Sedimentation equilibrium studies of the native enzyme and of the enzyme in 6 M guanidine HCl were carried out by Dr. Ann Ginsburg as follows: native protein (PIID) purified to electrophoretic homogeneity (initial concentration, A280 = 0.07) was dialyzed against 0.1 M potassium phosphate buffer, pH 7.3. The dialyzed protein was used as the reference solvent. Molecular weights were from slopes of linear plots of log A280 versus r2 (the square of the radial distance from the center of rotation) (28). Molecular weight calculations were based on a value of ε = 0.748 ml/g calculated from the amino acid analysis (24).

Sedimentation in 6 M Guanidine HCl—Because of the small subunit size, the protein in 6 M guanidine HCl was prepared as follows: after exhaustively dialyzing against 4 mM potassium phosphate, pH 7.2, the protein solution and an equal volume of 1 M sodium dodecyl sulfate were lyophilized. The lyophilized samples were each dissolved in 50 μl of dialysate to which 215 μl of 7.4 guanidine HCl were added, with the solution of 7.4 M guanidine HCl (Mann Research Laboratories, ultrapure grade) prepared by weighings; solvent densities accordingly were computed from the data of Kawahara and Tanford (25). The protein in 6 M guanidine HCl, with an initial concentration of A280 = 0.25, was run against the reference solvent (A280 = 0) for 24 hours at 30,000 and also at 33,000 rpm at 22°C.

Amino Acid Analysis—Amino acid analysis was carried out on a fluorescamine amino acid analyzer (20). Protein bands stained with Coomassie blue were sliced from acrylamide gels, lyophilized, and hydrolyzed in 6 N HCl, 1% thioglycolic acid for 24 hours at 110°C as described by Stein et al. (26, 27).

RESULTS

Procedure of Purification of Glutamine Synthetase Regulatory Protein Pm1 from Escherichia coli W

All of the following steps were carried out at 2-4°C. All additions were done with slow mechanical stirring. Distilled, deionized water was used throughout. Between steps, Pm1 was stored at -80°C. Activity was routinely followed by assaying Pm1 deadenylation activity even though the resulting preparation was at least 95% PIIA. The PIIA and Pm1 activities co-purified through all steps.
Step 1: Extraction—Cell-free extracts were prepared by homogenization of the frozen cell paste in 20 ml 2-methylimidazole, pH 7.6, 10 mM 2-mercaptoethanol, and 0.1 mM K3Mg EDTA (standard buffer; 2 liters of buffer per kilo of cell paste) in a Waring Blender for 90 s. The homogenized extract was filtered through cheesecloth and then passed two times through a French pressure cell at 12,000 p.s.i. Cell debris was removed by centrifugation for an hour at 12,000 × g. No PIIID activity could be detected at this step.

Step 2: Streptomycin-NH₄SO₄ Precipitation—A 10% solution of streptomycin sulfate was added slowly to the supernatant from Step 1 to a final concentration of 1%. After 15 min of slow stirring, the solution was centrifuged as above. To the supernatant, solid NH₄SO₄ was added to a final concentration of 300 mg/ml (50% saturated). After 30 min of stirring the precipitate was removed by centrifugation (15,000 × g for 15 min). The pellet was either stored frozen (−80°C) or immediately resuspended in a minimal volume of standard buffer and dialyzed two times against 200 volumes of this buffer. PIIID activity was first detectable at this stage. There was no loss of activity due to storage of the NH₄SO₄ pellet at −80°C.

Step 3: DEAE-cellulose Chromatography—The dialyzed extract from Step 2 was applied to a DEAE-cellulose column equilibrated with standard buffer containing 50 mM KCl. Approximately 33 mg of protein were applied per ml of bed volume. After removal of unabsorbed protein by washing with starting buffer, a linear KCl gradient (50 to 500 mM) in standard buffer was applied. Fractions were collected and assayed for their glutamine synthetase, adenylyltransferase, PII, and UR uridylyltransferase activities. Fig. 1 illustrates a typical elution profile from DEAE-cellulose. Glutamine synthetase and UR uridylyltransferase activities co-elute with the major protein peak at a salt concentration of approximately 75 to 100 mM KCl. This is the first step in the purification where UR uridylyltransferase activities are detectable. Under these conditions PII and adenylyltransferase co-elute from DEAE-cellulose at a KCl concentration of approximately 0.2 M. The fact that both adenylyltransferase and PII co-elute is probably not significant since these proteins can be readily separated by gel filtration, or by hydroxyapatite, or hydrophobic chromatography. The fractions containing the UR uridylyltransferase and PII adenylyltransferase activity peaks were pooled and concentrated by addition of (NH₄)₂SO₄ to 50% saturation as described in Step 2. The NH₄SO₄ precipitates were taken up in a minimal volume of standard buffer or standard buffer plus 100 mM KCl for UR uridylyltransferase factor, and stored at −80°C.

Step 4: Agarose Chromatography—Concentrated fractions (30 ml) containing PIII and adenylyltransferase were applied to an agarose column (5 × 120 cm) equilibrated with standard buffer. The column was then washed with standard buffer (100 cm of hydrostatic pressure), and 20-ml fractions were collected. After a void volume of approximately 600 ml had washed through the column, adenylyltransferase emerged along with the bulk of the protein. PII eluted just before the salt fraction. Only the highest specific activity tubes were pooled. A 30-fold purification of PII could be obtained at this step.

Step 5: Hydroxyapatite Chromatography—A column (3 × 15 cm) of hydroxyapatite (Bio-Rad HPT) was washed with approximately 2 liters of 5 mM potassium phosphate buffer, pH 7.0. Pooled fractions of PIII from the agarose column were applied to the hydroxyapatite column and was washed with 500 ml of 5 mM phosphate buffer. A linear gradient (5 to 100 mM potassium phosphate) of 1600 ml was then applied to the column. Ten-milliliter fractions were collected. PIII eluted at a phosphate concentration of approximately 40 mM. Fractions containing PIII activity were pooled and dialyzed two times versus 100 volumes of standard buffer.

Step 6: Hydrophobic Chromatography—Hydrophobic chromatography on Sepharose—(CH₂)₂₅NH₂ as described by Shaltiel (12) was used as a final purification step. It had previously been determined that (CH₂)₂₅NH₂ or (CH₂)₂₅NH₃ linked to Sepharose would completely retain PII and that 80 to 90% of the activity could be recovered by elution with KCl.2 Shorter hydrocarbon chain lengths did not retain all of the activity, whereas longer hydrocarbon arms ( > CH₂) retained all of the activity but elution with simple salt solutions was not efficient. Thus, a column (1.5 × 8.5) of Sepharose—(CH₂)₂₅NH₂ was prepared (see “Experimental Procedures”) and equilibrated with standard buffer. The dialyzed material from hydroxyapatite (10 to 100 ml, 0.1 mg/ml)

![Fig. 1. Chromatography of GS, PII, adenylyltransferase, UR, and uridylyltransferase on DEAE-cellulose (DES II Whatman); 70 ml of dialyzed extract (40 mg/ml) were applied to a DEAE-cellulose column (2.5 × 27 cm) equilibrated with 20 mM 2-methylimidazole-HCl, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 50 mM KCl, pH 7.63. After the unabsorbed protein was eluted, a linear KCl gradient (50 to 500 mM KCl) in the above buffer was applied. Protein was monitored by absorbance at 280 nm (after diluting 1 to 5). Salt concentration was monitored by conductivity. Ten-milliliter fractions were collected.](http://www.jbc.org/)
Table I

Purification summary

| Step                          | Activitya | Protein | Specific activity |
|-------------------------------|-----------|---------|------------------|
|                               | pmol AMP released/ min/ml | mg/ml | pmol AMP released/ min/mg |
| Crude                         | 535       | 74.1    | 7                |
| Streptomycin (10%)            | 1,739     | 51.1    | 34               |
| NH₄SO₄ (0–50%)                | 1,539     | 1.48    | 1,040            |
| DEAE-cellulose chromatography | 750       | 0.114   | 6,584            |
| Hydroxypatite chromatography  | 750       | 0.114   | 6,584            |
| Hydrophobic chromatography    | 472       | 0.04    | 11,808           |

* Activity assayed on the basis of deadenylylation activity (P₁₁₁) where the rate of release of [³⁵S]AMP from glutamine synthetase is measured.

was applied to the column and the column was washed with a linear KCl gradient (0 to 0.5 M; 400 ml) in standard buffer. P₁₁ activity eluted at approximately 0.2 M KCl. Peak activity fractions were stored at -80° and used for analytical studies.

Table I summarizes a typical preparation. The over-all yield was calculated to be approximately 10%. The P₁₁ at all stages of purification was stable for at least 6 months when stored at -80° in standard buffer. The same procedure was used to purify P₁₁ from Pseudomonas putida except that Step 6 was not necessary to obtain a homogeneous preparation.

Molecular Weights of P₁₁₁ and P₁₁₁₁

The form of P₁₁ isolated as described above was the unmodified or P₁₁₁₁ form. It migrated as a single protein band when subjected to electrophoresis in 7.5% polyacrylamide gels (Fig. 2A) or in gels containing 0.1% sodium dodecyl sulfate. Conversion of the P₁₁₁₁ to the fully uridylylated form by incubation in the presence of [³⁵S]UTP and uridylyltransferase followed by repurification over hydroxyapatite changed its electrophoretic mobility in standard gels (Fig. 2C). A mixture of the two forms of P₁₁, the unmodified form (P₁₁₁₁) and the fully uridylylated form (P₁₁₁₁₁) was completely separated electrophoretically (Fig. 2B). To determine whether this difference in electrophoretic mobilities between the two forms was due primarily to size or charge differences, the molecular weights of both species were determined by electrophoresis in gels of varying porosities (Fig. 3). Ribonuclease, ovalbumin (monomer and dimer), and bovine serum albumin; RNase, ribonuclease A. The subscripts 1, 2, and 3 denote monomer, dimer, and trimer.

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Subunit Molecular Weight

The subunit molecular weight of P₁₁ as determined electrophoretically in sodium dodecyl sulfate acrylamide gels is 11,000.

1 C. Huang, and S. P. Adler, unpublished data.
A value of 11,600 was calculated from electrophoretic mobilities in the presence of 0.1% sodium dodecyl sulfate and chloramine-T prior to tryptic digestion of the protein. A minimal molecular weight of 11,350 was calculated from the amino acid content, Table II. Based upon these data the protein appears to be composed of four identical subunits.

There are 2 tyrosyl residues (Table II) per minimal molecular weight species and if these 2 residues are separated by a trypsin-susceptible bond and they are four subunits identical, then tryptic digestion should yield equimolar amounts of two tyrosine-containing peptides. To facilitate detection of such peptides a PIIA preparation was iodinated with $^{35}$I in the presence of 0.1% sodium dodecyl sulfate and chloramine-T prior to tryptic digestion as described by Bray and Brownlee (30). As shown in the upper part of Fig. 4 only two radioactive peptides were formed. When the radioactive spots containing these peptides were cut from the paper and counted in a liquid scintillation spectrometer, one contained 124,100 cpm and the other 123,590 cpm. The fact that two tryptic peptides containing tyrosine are present in equal molar amounts is further evidence that the subunits are identical.

Site of UMP Linkage

When fully uridylylated PII (PIII) was iodinated, digested with trypsin, and the radioactive peptides separated by the identical procedure used for PIIA, only one of the two radioactive peptides produced from PIIA was detected (lower part of Fig. 4). This suggested that the uridyl group was covalently attached to the hydroxyl oxygen of one of the tyrosine residues since a free hydroxyl group is essential for the iodination of tyrosine under these conditions. In control experiments free tyrosine was shown to be readily iodinated by the chloramine-T method, whereas O-phosphotyrosine was not iodinated. Thus, the fact that only one of the two tyrosyl groups of a uridylylated PII subunit can be iodinated indicates that the UMP is covalently attached to the hydroxyl group of the other tyrosyl residue in each of the subunits. Iodination and tryptic digestion of partially uridylylated PII yields varying amounts of the iodinated tyrosyl peptide that is not detected in digests of fully uridylylated PII (data not shown).

With the exception of the amino acid analyses, which show minor differences in composition between the E. coli PII and the P.putida PII, the PII preparations from the two microorganisms are otherwise indistinguishable by the methods described in the present study. Hydrolysis of the P.putida PII yielded two tyrosyl peptides with map positions identical with those obtained from the E. coli protein.

Independent evidence supporting the conclusion that UMP is linked to PI through the hydroxyl group of tyrosine is shown in Fig. 5. When a difference spectrum at pH 13 was taken between PIID (in the reference cuvette) and PIID treated with snake venom phosphodiesterase to remove the covalently bound UMP (in the sample cuvette), a 290-nm absorption peak characteristic of an ionized tyrosyl hydroxyl group was observed. In a comparable experiment, incubation of [3H]UMP-uridylylated PII (112,000 cpm/mol) with phosphodiesterase resulted in the release of 5.1 µmol [3H]UMP and the concomitant appearance of 5.5 µmol ionizable tyrosyl groups (calculated from a molar extinction coefficient of 2,330, pH 13). Thus, there is an approximately 1:1 molar relationship between the amount of UMP cleaved by phosphodiesterase and ionizable tyrosyl groups exposed.

A summary of the physical properties of PII described above is shown in Table III.

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**Table II**

Amino acid composition of PIIA (Escherichia coli)

| Amino acid  | Sample 1 | Sample 2 | Nearest Integer |
|-------------|----------|----------|----------------|
| Lysine      | 9.65     | 8.88     | 9              |
| Histidine   | 0.83     | 0.97     | 1              |
| Arginine    | 6.19     | 6.93     | 7              |
| Aspartic acid | 10.9    | 11.2     | 11             |
| Threonine   | 7.1      | 7.06     | 7              |
| Serine      | 0.68     | 1.27     | 1              |
| Glutamic acid | 9.55   | 9.8      | 10             |
| Proline     | 3.19     | 3.66     | 4              |
| Glycine     | 10.7     | 10.8     | 11             |
| Alanine     | 6.96     | 6.7      | 7              |
| Valine      | 13.0     | 11.2     | 12             |
| Methionine  | 2.28     | 1.91     | 2              |
| Isoleucine  | 11.1     | 10.4     | 11             |
| Leucine     | 4.03     | 4.09     | 4              |
| Tyrosine    | 1.80     | 1.78     | 2              |
| Phenylalanine | 4.80   | 4.32     | 5              |
| Cysteic acid| 0        | 0        | 0              |

* The authors are indebted to Dr. Stanley Stein for his assistance in carrying out the amino acid analysis.
FIG. 5. Difference spectrum between deuridylylated and uridylylated 72 s at pH 13. $p_{12}$ purified from Pseudomonas putida (133 $ug/ml$) in 10 mm Tris-HCl, pH 8.0, was supplemented with 10 mm MgCl$_2$ and added to each of two matched cuvettes (1 ml). One microliter of snake venom phosphodiesterase (3 mg/ml, Worthington) was added to the sample cuvette. Following incubation at 37° for 3 hours both solutions were made 20 mm in Na EDTA. Snake venom phosphodiesterase was then added to the reference cuvette and both solutions were made 0.10% in sodium dodecyl sulfate and 0.1 $M$ in NaOH. The difference spectrum was then recorded in a Cary 17 recording spectrophotometer equipped with a 0 to 0.1 slide wire. The solution that had been incubated with phosphodiesterase was in the sample position.

FIG. 6. Chromatography of uridylyltransferase and uridylyl-removing activity on Sepharose-(CH$_2$)$_5$-NH$_2$. 4 ml (40 mg/ml) of protein solution containing UR and uridylyltransferase activity previously isolated from DEAE-cellulose as described under “Results” were applied on a Sepharose-(CH$_2$)$_5$-NH$_2$ column (8.5 X 11.5) equilibrated at 4° with 20 mm 2-methylimidazole, 100 mm KCl, 10 mm 2-mercaptoethanol, and 0.1 mm EDTA, pH 7.6. After the unabsorbed protein was eluted a linear KCl gradient was applied up to 0.4 $M$. Protein was monitored by absorbance at 280 nm and salt concentration by conductivity. 5.2 ml fractions were collected and assayed for their UR and uridylyltransferase activities. B, fractions 50 to 75 from A were pooled and concentrated by NH$_4$SO$_4$ precipitation as above. 5.5 ml (17 mg/ml) of this material was reapplied to the same column in A which had been equilibrated with the above starting buffer. A linear KCl gradient up to 0.3 $M$ KCl was applied.

Properties of $p_{12}$ regulatory protein

| Property                        | Value       | Method obtained                                      |
|--------------------------------|-------------|-----------------------------------------------------|
| Molecular weight               | 44,000      | Ferguson plot-acrylamide gels                        |
|                                | 41,700 ± 2,300 | Sedimentation equilibrium                            |
| Subunit molecular weight       | 11,000      | Sodium dodecyl sulfate acrylamide gels               |
|                                | 11,600      | Ferguson plot-8 $M$ urea acrylamide gels             |
|                                | 13,400 ± 2,000 | Sedimentation equilibrium in 6 $M$ guanidine HCl   |
|                                | 11,350      | Amino acid composition                               |
| UMP linkage to hydroxyl of tyrosine |            | Ultraviolet spectroscopy (pH 13)                    |
| Moles of UMP bound per tetramer | 4          | Differential iodination of uridylylated and non-uridylylated tryptic peptides |

* Determined at 15,000 rpm (see “Experimental Procedures”). Approximately 8% dissociation of the tetramer to the dimer was apparent in the scans at 28,000 rpm.

Properties of Uridylyltransferase-Uridylyl-removing Enzyme

Activities of the enzyme(s) responsible for the covalent attachment and subsequent removal of the uridylyl group from the $p_{12}$ regulatory protein are not detectable in crude extracts. This is presumably due to an inhibitor present in the initial extracts which is separated from these activities by DEAE-cellulose chromatography, although the nature of this inhibitor has not been determined. As illustrated in Fig. 1, the uridylyltransferase and uridylyl-removing enzyme activities co-elute from DEAE-cellulose. Both the UR and uridylyltransferase activities co-purify through a variety of subsequent procedures including: gel filtration, hydroxyapatite chromatography, isoelectric focusing, sucrose density gradient centrifugation, hydrophobic chromatography and polyacrylamide gel electrophoresis. An example of their co-purification is illustrated in Fig. 6. When the DEAE-cellulose fractions containing UR and uridylyltransferase activities from the first passage on Sepharose-(CH$_2$)$_5$-NH$_2$ were pooled, concentrated, and reapplied to the same column, elution with a shallower KCl gradient again failed to resolve the two activities and a second minor peak of both activities appeared (Fig. 6B). Whereas the UR uridylyltransferase activities had been extensively purified, a homogeneous preparation has not been obtained.

Both the UR and uridylyltransferase activities are heat labile in the absence of high ionic strength buffers. Incubation at 37° for 30 min in low ionic strength buffers destroys both activities but addition of KCl (100 mm) completely protects against this inactivation. Routinely, 100 mm KCl is used in all buffers and assay mixtures, although other salts except phosphate (which inhibits both activities) of equal ionic strength can replace KCl. These include NaCl, Na$_2$SO$_4$, K$_2$SO$_4$, MgCl$_2$, CaCl$_2$, and MgSO$_4$. Under the usual assay conditions ("Experimental Procedures") uridylyltransferase had a pH optimum of 7.6, whereas UR activity was greatest between pH 8.6 and 9.0.

The effects of divalent cations and other effectors on the UR and uridylyltransferase activities are shown in Table IV. Mo$_{2+}$ by itself can support maximal UR activity, whereas Mg$_{2+}$ cannot support this activity except in the presence of $\alpha$-ketoglutarate.
TABLE IV

Effect of divalent cations and other effectors on UR and uridylyltransferase activities

| Effectors added | UR activity | Uridylyltransferase activity |
|-----------------|-------------|-----------------------------|
| Mn²⁺, ATP, aKG  | 85%         | 100%                        |
| Mn²⁺, ATP       | 85%         | 35%                         |
| Mn²⁺, aKG       | 100%        | 44%                         |
| Mn²⁺           | 100%        | 0%                          |
| Mn²⁺, Mg²⁺     | 100%        | 0%                          |
| Mg²⁺, ATP, aKG | 55%         | 100%                        |
| Mg²⁺, ATP       | 27%         | 27%                         |
| Mg²⁺, aKG       | 25%         | 33%                         |
| Mn²⁺, glutamine| 100%        | 0%                          |
| Mg²⁺, ATP, aKG, glutamine | 55% | 7% |

* Reaction mixtures also contain 2 mM K₃Mg EDTA to chelate traces of Mn²⁺ that were present in the enzyme preparation.

Effect of divalent cations and other effectors on UR and uridylyltransferase activities

and ATP; even then the activity with Mg²⁺ is less than with Mn²⁺. In contrast, Mn²⁺ and Mg²⁺ support equal uridylyltransferase activity in the presence of ATP and α-ketoglutarate but neither divalent cation is able to support activity in the absence of these effectors. Table IV shows further that uridylyltransferase is strongly inhibited by glutamine, whereas glutamine does not affect UR activity. Thus, the fact that both UR and uridylyltransferase activities co-purify, are stabilized by high ionic strength buffers, and in the presence of Mg²⁺ require α-ketoglutarate and ATP for activity suggests that a single enzyme or enzyme complex may catalyze both reactions.

Relationship between Uridylation and Capacity of PII to Stimulate Adenylylation or Deadenylylation of Glutamine Synthetase

As predicted from the data of Table IV, the UR uridylyltransferase preparation, in the absence of all effectors except Mn²⁺, catalyzes a rapid release of the covalently bound UMP groups from [³H]UMP-PIID (Fig. 7). Moreover, cleavage of the UMP groups from PII is accompanied by a parallel increase in its capacity to stimulate the adenylyltransferase-catalyzed adenylylation of glutamine synthetase and a concomitant loss in its ability to stimulate adenylyltransferase-catalyzed deadenylylation of glutamine synthetase. These reciprocal changes in PII in the presence of Mn²⁺ only. Nevertheless, it seemed likely that UMP was the primary product since a rapid hydrolysis of UMP to uridine was catalyzed by the UR enzyme preparation. Using a more highly purified UR enzyme prepared as described in Fig. 7, UMP was identified as the primary cleavage product either in the presence of Mn²⁺ or with Mg²⁺, ATP, and α-ketoglutarate (Table V). Addition of UMP (Line 2 of Table V) had no effect upon this reaction.

Identification of UMP as Cleavage Product

Previous reports from this laboratory (8) had shown that uridine rather than UMP was the major product to accumulate when [¹⁴C]UMP-PIID was treated with UR enzyme in the presence of Mn²⁺ only. Nevertheless, it seemed likely that UMP was the primary product since a rapid hydrolysis of UMP to uridine was catalyzed by the UR enzyme preparation. Using a more highly purified UR enzyme prepared as described in Fig. 7, UMP was identified as the primary cleavage product either in the presence of Mn²⁺ or with Mg²⁺, ATP, and α-ketoglutarate (Table V). Addition of UMP (Line 2 of Table V) had no effect upon this reaction.
TABLE V

Product of UR-catalyzed deuridylylation of [3H]UMP.

| Effectors added | Product formed | cpm (%% total) |
|-----------------|---------------|---------------|
| [3H]UMP         | UMP           | Uridine       |
| Mg++, ATP, αKG  | 994 (20)      | 3543 (74)     | 240 (9)     |
| Mg++, ATP, UMP  | 1206 (23)     | 3703 (73)     | 183 (4)     |
| Mn++, None      | 659 (12)      | 4402 (84)     | 211 (4)     |
| None            | 7407 (97)     | 109 (1)       | 149 (2)     |

*These reaction mixtures also contain 2 mM K₃Mg EDTA to chelate traces of Mn²⁺ that were present in the enzyme preparation.

Fig. 8 summarizes current knowledge of the complex system that regulates glutamine synthetase activity in E. coli. It consists essentially of two enzyme-catalyzed interconversion systems that are interconnected by the PII regulatory protein. The uridylylation system involves covalent attachment and detachment of UMP to and from the regulatory protein; this in turn regulates the adenylylation and deadenylylation of glutamine synthetase and thereby determines its catalytic potential. Viewed in a more conventional manner, the covalent modification systems constitute two opposing cascade systems that lead either to the activation or inactivation of glutamine synthetase. As shown in Fig. 9B, inactivation of glutamine synthetase activity is initiated by the activation of the UR enzyme which catalyzes the conversion (diuridylylation) of PIA to PIIA. The latter, presumably by direct action, stimulates the capacity of adenylyltransferase to catalyze the adenylylation of glutamine synthetase, thus converting it from a Mg²⁺-dependent form with a pH optimum of 8.0 to the less active Mn²⁺-dependent form having a pH optimum of 6.9. A similar cascade leading to the activation of glutamine synthetase is depicted in Fig. 9A. This cascade is initiated by the action of uridylyltransferase which catalyzes the uridylylation of PIIA, converting it to the modified form, PIID, whose interaction with adenylyltransferase stimulates the deadenylylation of glutamine synthetase, which is then converted back to the more active Mg²⁺-dependent form. As shown in both Figs. 8 and 9, the activities of the uridylylation and adenylylation systems and thus glutamine synthetase activity are finely modulated by the concentrations of various metabolites including UTP, ATP, α-ketoglutarate, PII, glutamine, and probably other compounds as yet unidentified.

The PII regulatory protein has a molecular weight of approximately 44,000 and has four identical subunits, each of which contains a specific tyrosyl residue that is covalently modified by the attachment of UMP in a phosphoryl-O-tyrosyl linkage. The role of this uridylylation in the conversion of PIIA to PIID has been clearly demonstrated. The fully uridylylated protein (PIIA) can be separated from the unmodified form (PIIA) by polyacrylamide gel electrophoresis on the basis of charge, presumably due to the phosphoryl groups of PIID. Since each subunit can be uridylylated, there are at least five species of PII that differ in the number (0 to 4) of moles of UMP bound per mole of protein. Preliminary results indicate that at least four and perhaps five species are resolved by gel electrophoresis. Since the relative mobilities of these species are altered by treatment with either snake venom phosphodiesterase (which deuridylylates PIID) or with uridylyltransferase, it is probable that they differ from one another by the...
number of covalently bound uridylyl groups. It remains to be determined whether the regulation characteristics of hybrid protein molecules are affected by heterologous interactions between uridylylated and deuridylylated subunits.

The activities responsible for the uridylylation and deuridylylation of P11 have been partially purified and characterized. In the studies described in this communication all attempts to separate the UR and uridylyltransferase activities failed. However, Mangum et al. (8) obtained a uridylyltransferase preparation containing no UR activity, and later work has also yielded some uridylyltransferase fractions containing little or no UR activity, but UR enzyme preparations free of uridylyltransferase have never been obtained. If both activities are catalyzed by the same enzyme or enzyme complex, these results indicate that the UR moiety can be differentially lost either by dissociation or partial denaturation of the complex. Both activities are, however, heat labile in the absence of high ionic strength buffers.

As previously observed (8) Mn²⁺ alone will support UR activity but not uridylyltransferase activity. However, in the presence of ATP and α-ketoglutarate, Mg²⁺ and Mn²⁺ will support both UR and uridylyltransferase activities. It is, therefore, possible that metal ions as well as metabolites play an important role in regulating these activities. Another possibility is that Mg²⁺ is the physiologically important intracellular cation and that the Mn²⁺-supported activity is without physiological importance. This, however, would mean that ATP and α-ketoglutarate are positive effectors for not only deadenylation but also for adenylation, since their activation of UR activity to produce P11 would lead to stimulation of adenylation. Thus, it is possible that the Mn²⁺ and the ATP, α-ketoglutarate, and Mg²⁺-supported UR activities are catalyzed by different sites either on the same or different proteins, and that the UR activity supported by Mg²⁺, ATP, and α-ketoglutarate which is only 55% as active as Mn²⁺-supported UR activity (Table IV) is physiologically unimportant and may even be a partial reversal of the uridylyltransferase reaction. These possibilities remain to be explored.

Because they are present in relatively low concentrations, studies of the P11 and UR uridylyltransferase activities have been hampered by the inability to isolate sufficient quantities of homogeneous proteins. It is fitting that these proteins are present in low intracellular concentrations since they are components of a cascade system. In such systems the maximum and most efficient amplification of input signals (in this case changes in the concentration of various metabolites including ATP, α-ketoglutarate, and glutamine, etc.) is achieved when the concentrations of the modifying enzymes decrease progressively the further removed their activity is from that of the target enzyme, glutamine synthetase. Thus, the concentration of UR uridylyltransferase should be significantly less than P11 or adenyllyltransferase, and these should be present in lower concentrations than glutamine synthetase. This, in fact, appears to be the case. P11 was purified 1600-fold to homogeneity. Although UR uridylyltransferase has not been obtained in a homogeneous form, preliminary results indicate a significantly greater extent of purification will be necessary. In contrast, glutamine synthetase is present in very high intracellular concentrations and requires only 100- to 200-fold purification. A further difficulty in the isolation of these proteins has been the inability to accurately assay their activities in crude extracts. UR uridylyltransferase activity cannot be detected prior to DEAE-cellulose chromatography. P11 is not accurately assayable in crude extracts, because its activity is known to be influenced not only by endogenous adenyllyltransferase but also by endogenous inhibitors of deadenylation activity known to be present (31). In these respects it should be noted that P11 activity in crude extracts of P. putida is at least 40-fold higher than in E. coli grown under identical conditions and homogeneous P11 preparations are obtained after only 120-fold purification. Since the P. putida P11 appears to have the same regulatory and physical properties as the E. coli protein, this may be a more suitable source of P11 for further detailed studies.

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