Enzymological Characterization of the Signal-Transducing Uridylyltransferase/ Uridylyl-Removing Enzyme (EC 2.7.7.59) of *Escherichia coli* and Its Interaction with the PII Protein†

Peng Jiang, James A. Peliska, and Alexander J. Ninfa*

Department of Biological Chemistry, University of Michigan Medical School, 1301 East Catherine, Ann Arbor, Michigan 48109-0606

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ABSTRACT: The uridylyltransferase/uridylyl-removing enzyme (UTase/UR) of *Escherichia coli* plays an important role in the regulation of nitrogen assimilation by controlling the uridylylation state of the PII signal transduction protein (PII) in response to intracellular signals. The reversible uridylylation of PII indirectly controls the activity of PII receptors that regulate transcription from nitrogen-regulated promoters and the activity of glutamine synthetase. Here, we present a detailed analysis of the uridylyltransferase and uridylyl-removing activities and their regulation by the small molecule effectors ATP, 2-ketoglutarate, and glutamine. Several important features of enzyme mechanism and regulation were elucidated. Mg^{2+} appeared to be the physiologically relevant metal ion cofactor for both transferase and uridylyl-removing activities. The transferase reaction proceeded by an ordered bi−bi kinetic mechanism, with PII binding before UTP and pyrophosphate (PPi) released before PII−UMP. The uridylyl-removing reaction proceeded with rapid equilibrium binding of substrate and random release of products. Both reactions were activated by ATP and 2-ketoglutarate, which did so by binding only to PII and PII−UMP. The binding of these effectors to PII and PII−UMP was characterized. Glutamine inhibited the transferase reaction by inhibiting the chemistry step, while glutamine provided nonessential mixed-type activation of the uridylyl-removing activity, lowering the apparent K_m and increasing k_{cat}. Our data were consistent with the hypothesis that all effects of glutamine are due to the binding of central complexes at a single glutamine site. By comparing the effects of the activators with their reported in vivo concentrations, we conclude that in intact cells the uridylylation state of PII is regulated mainly by the glutamine concentration and is largely independent of the 2-ketoglutarate concentration. Our kinetic data were consistent with the hypothesis that both transferase and uridylyl-removal reactions occurred at a single active center on the enzyme.

The signal-transducing uridylyltransferase/uridylyl-removing enzyme (EC 2.7.7.59) of *Escherichia coli* and related bacteria, product of the *glnD* gene, plays a key role in the regulation of glutamine synthetase activity and biosynthesis by virtue of its interactions with the PII signal transduction protein[1] (reviewed in refs *1*−*3*). Under conditions of nitrogen starvation, the UTase/UR enzyme catalyzes the conversion of the PII protein and UTP to PII−UMP and PPi (*4*−*10*). Previous studies with the purified enzyme indicated that this reaction requires either Mg^{2+}, 2-ketoglutarate, and ATP (*6*), which bind with high affinity to the PII protein (*10*), or Mn^{2+} and ATP (*6*). In either case, the transferase activity is inhibited by glutamine (*6*), which does not apparently bind to the PII protein (*10*) and thus must interact with the enzyme. Conversely, under conditions of nitrogen excess, PII−UMP is converted to PII and UMP in a hydrolysis reaction that is greatly stimulated by either Mn^{2+} and glutamine or Mg^{2+}, glutamine, ATP, and 2-ketoglutarate (*6*−*8*). The interconversion of the PII protein between uridylylated and nonuridylylated forms constitutes one of the most important mechanisms for signaling of the intracellular nitrogen status. PII, but not PII−UMP, stimulates the phosphatase activity of nitrogen regulator II (NRII, NtrB), the kinase/phosphatase responsible for the regulation of transcription of nitrogen-regulated genes (*11*−*13*). Both PII and PII−UMP are regulators of adenylyltransferase (ATase), responsible for the regulation of glutamine synthetase (GS) activity by reversible covalent modification (reviewed in ref *3*). PII stimulates the adenylylation (inactivation) of GS, while PII−UMP is required for the deadenylylation (activation) of GS−AMP.

While the overall requirements for the uridylylation and deuridylylation of the PII protein by the UTase/UR enzyme have been known for some time, the mechanisms of the uridylyltransferase and uridylyl-removing activities, along with the mechanisms of their regulation, remain largely unknown. For example, until recently it was thought that 2-ketoglutarate and ATP bound to the UTase/UR enzyme (*3*), as opposed to their actual role as ligands of the PII

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* Author to whom correspondence should be addressed. Phone: 734-763-8065. Fax: 734-763-4581. E-mail: aninfa@umich.edu.

[1] Abbreviations: UTase/UR, uridylyltransferase/uridylyl-removing enzyme; PII, PII signal transduction protein; NRI, nitrogen regulator I or NtrC; NRII, nitrogen regulator II or NtrB; GS, glutamine synthetase; ATase, adenylyltransferase; PPi, pyrophosphate; PEI, polyethyleneimine.

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protein (*10*). Furthermore, it has been proposed that the uridylylation state of PII is regulated by the ratio of the intracellular glutamine and 2-ketoglutarate concentrations (*1*, *3*, *7*) and that the UTase and UR reactions might occur at separate sites on the enzyme (*3*). We will show here that both of these hypotheses are not consistent with our data. Part of the reason the uridylyltransferase/uridylyl-removing enzyme has remained enigmatic is that until recently stable preparations of the enzyme were not available (*6*−*8*). Recently devised methods for the hyperexpression and purification of the enzyme (*9*), resulting in stable, essentially homogeneous enzyme, have permitted the detailed characterization of the enzyme reported here.

Sequence analysis of the *glnD* gene (*14*) indicates that the N-terminal portion of the UTase/UR is related to an ancient family of nucleotidylyltransferase proteins that include DNA polymerase β from the eucaryotic "X" family of DNA polymerases (EC 2.7.7.4), kanamycin nucleotidylyltransferase (EC 2.7.7), poly(A) polymerase (EC 2.7.7.19), DNA nucleotidylyl exotransferase (EC 2.7.7.31), streptomycin 3′-adenylyltransferase (EC 2.7.7.47), *E. coli* glutamine synthetase adenylyltransferase (ATase, EC 2.7.7.42), and several other enzymes (*15*). The structures of two of these related nucleotidylyltransferase enzymes, kanamycin nucleotidylyltransferase and DNA polymerase β, have been determined (*16*−*20*); these share a remarkable conservation of structure within their catalytic domains suggesting a common mechanism of catalysis. In the case of DNA polymerase β, the polymerization reaction is distributive (nonprocessive), and occurs by a sequential ordered bi−bi mechanism in which the template DNA substrate binds prior to dNTP binding, and PPi release precedes the release of the extended DNA chain (*21*). The only available information concerning the UTase/UR kinetic mechanism is contained in a single article (*3*), where the reaction is described as ordered, with nucleotide binding first. In the work reported here, we examined the steady-state kinetics of the uridylyltransferase and uridylyl-removing reactions, the activation and inhibition of these activities by small molecule effectors, and other properties of the enzyme, including the nucleotide substrate specificity.

MATERIALS AND METHODS

Purified Proteins. UTase/UR and PII proteins were the generous gift of Mariette R. Atkinson and were purified and stored as described previously (*9*). The construction and purification of mutant forms of the PII protein was described (*22*); the protein preparations from that study were used. PII−UMP was purified as described previously (*11*), and purified fractions were subjected to a brief heat treatment (60 °C, 10 min) to destroy any contaminating UTase/UR activity. Nondenaturing gel electrophoresis of PII−UMP was performed as described (*11*) and indicated that the purified PII−UMP was >90% uridylylated. Similar methods were used to purify and characterize PII−CMP and PII−AMP. Adenylyltransferase (ATase) and adenylylated glutamine synthetase were the generous gift of Priya Chandran and were purified as described previously (*23*, *24*). Nitrogen regulator I (NRI) and nitrogen regulator II (NRII) were the generous gift of Emmanuel Kamberov and were purified as described previously (*9*). Proteins concentrations were determined by the method of Lowry (*25*) using BSA as the standard. Previous work using N-terminal sequencing and/or amino acid analysis of these protein has indicated that the Lowry assay provides an accurate estimation of protein concentration for these proteins (A. J. N., E. S. Kamberov, and M. R. Atkinson, unpublished data). The concentrations of PII and mutant PII proteins are stated as the trimer, the concentrations of the UTase/UR and ATase are stated as the nominal momomer concentration, and the concentrations of NRI and NRII are stated as the dimer.

Uridylyltransferase Assay. The assay is a modification of that reported previously (*11*). Reaction mixtures were assembled on ice containing 100 mM Tris-Cl, pH 7.5, 100 mM KCl, 25 mM $MgCl_2$, 0.5 mM ATP (or as indicated), 33 μM 2-ketoglutarate (or as indicated), 1 mM DTT, 0.3 mg/mL BSA, 4 nM UTase/UR enzyme (or as indicated), and PII as indicated. The reaction mixtures were preincubated at 30 °C for 2 min, then [α-^{32}P]UTP (or [α-^{32}P]-labeled CTP, GTP, or ATP, as indicated) was added to the indicated concentration and the incubation at 30 °C was continued. Aliquots (4 μL) were removed at the indicated times and spotted onto nitrocellulose filters (Schleicher and Schuell BA85), which were immersed immediately and washed extensively in 5% trichloroacetic acid. Acid-insoluble radioactivity was determined by liquid scintillation in a Beckman LS380 instrument, using Fisher Scintiverse scintillant. For Mn^{2+}-UTase and Mn^{2+} + Mg^{2+}-UTase assays, conditions were as above except that 1 mM $MnCl_2$ or 1 mM $MnCl_2$ + 10 mM $MgCl_2$ replaced the $MgCl_2$, respectively, and 2-ketoglutarate was omitted unless indicated.

To examine the rate of formation of the PPi produced by the transferase reaction, we enzymatically synthesized γ-[^{32}P]-UTP from UDP and γ-[^{32}P]ATP, using nucleoside diphosphate kinase (NDK, Sigma Chemical Co.). Conditions for the NDK reactions were 50 mM Tris-Cl, pH 7.5, 25 mM $MgCl_2$, 100 mM KCl, 1 mM UDP, 0.334 μM [γ-^{32}P]ATP, and 0.45 units of NDPK in a volume of 50 μL. After incubation of the reaction mixture for 15 min at 30 °C, NDPK was removed by centrifugal ultrafiltration of the sample in a Microcon-10 apparatus (Amicon) at 4 °C, 3000 rpm, using a microfuge. Thin-layer chromatography (see below) of the product indicated essentially complete transfer of the ^{32}P label to UTP (not shown). This centrifugal ultrafiltration treatment completely removed NDPK, as shown by testing an aliquot of the reaction mixture for the production of labeled ATP upon addition of ADP to 10 mM, followed by analysis by thin-layer chromatography (not shown).

To measure the rate of formation of PPi, transferase reactions were performed as above, except that [γ-^{32}P]UTP was used, and aliquots were removed at various times and mixed with 50 mM EDTA to stop the reactions. Aliquots (1 μL) of the reaction mixtures were then subjected to thin-layer chromatography (see below), and the radioactivity in the position corresponding to PPi was estimated by phosphoimaging (PhosphorImager model 445SI, Molecular Dynamics).

To measure the rate of the reversal of the transferase reaction (the pyrophosphorolysis of PII−UMP), conditions were as in the transferase reaction except that PII and UTP were omitted, enzyme was present at 0.5 μM, [^{32}P]PPi was 0.5 mM, PII−UMP was 20 μM, $MgCl_2$ was 25 mM, and 2-ketoglutarate was 1 mM. After incubation of the reaction

mixtures at 30 °C for various times, 4 μL samples were removed and combined with an equal volume of 0.2 M EDTA to stop the reactions. Aliquots of the samples were subjected to thin-layer chromatography, and the chromatograms were subjected to autoradiography to locate the UTP spots, which were excised and counted by liquid scintillation. To examine the effect of glutamine on the reversal of the transferase reaction, conditions were as in the standard transferase reaction except that [^{32}P]PPi was present at 2.5 mM, PII−UMP was 10 μM, enzyme was 0.5 μM, UTP was 0.1 mM, and glutamine, when present, was 20 mM. Samples were incubated and treated as described above.

To examine whether either product demonstrated burst accumulation in the presence of glutamine, reactions were performed as described above, except that high concentrations of the enzyme (ranging from 0.5−4 μM) were used, PII was 10 μM, ATP was 0.15 mM or 0.5 mM, and 2-ketoglutarate was 1 mM. In experiments investigating PPi production in the presence of 20 mM glutamine, γ-[^{32}P]UTP was present at 50 μM to ensure that a burst equivalent to the enzyme concentration would be evident.

Substrate inhibition studies were essentially as described above, except that PII concentration was varied over a wide range. Substrate inhibition studies were not conducted with the UTP substrate, since initial pilot studies indicated no inhibition even at very high UTP concentrations.

Data Analysis. Initial velocity data were obtained in two ways; in both cases the raw data was corrected by subtraction of the background (i.e., counts adhering to filters that were washed simultaneously but had received no sample). First, the data for the initial stages of the reaction (<10% of the reaction progress), which look linear to the eye, were fitted by linear regression using the Enzfit program, and second, data were plotted manually and initial rates were estimated visually using a ruler. Both methods gave similar values for the initial rates. Initial rates were then analyzed by the KinetAssist program for fitting to various kinetic models by nonlinear regression methods. To fit the data to a sequential reaction mechanism (Figure 1), the equation used was: $v = V_mAB/(K_{ia}K_b + K_aB + K_bA + AB)$. For fitting inhibition patterns, the following equations were used: Competitive inhibition, $v = V_mS/[K_m(1 + I/K_{is}) + S]$; uncompetitive inhibition, $v = V_mS/[K_m + S(1 + I/K_{ii})]$; noncompetitive inhibition, $v = V_mS/[K_m(1 + I/K_{is}) + S(1 + I/K_{ii})]$; where S is the concentration of substrate and I is the concentration of inhibitor.

Optimization of the Uridylyltransferase Assay. We verified that UTase activity is stable at the conditions and within the time scale used in the experiments performed here (data not shown). The recovery of the PII−UMP product in reaction mixtures containing various concentrations of BSA was examined. These experiments were motivated by the observation in preliminary experiments that, in the absence of BSA, recovery of product in reactions containing a low concentration of PII (2 μM) was considerably less than anticipated, when compared to experiments conducted with PII concentrations of 10 μM or higher (data not shown). To determine the optimal BSA concentration, assay mixtures contained 2 nM UTase/UR, 2 μM PII, 500 μM UTP, and various BSA concentrations. Optimal conditions for the recovery of PII−UMP product was found to be 300 μg/mL BSA (data not shown); product recovery was improved

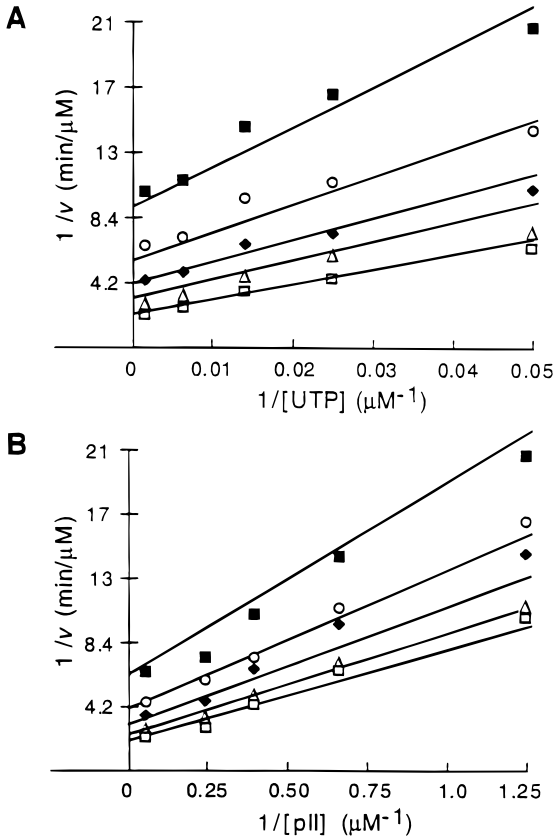

FIGURE 1: Substrate initial velocity patterns. Shown are Lineweaver−Burke transformations with UTP (panel A) and PII (panel B) as varied substrate. The PII concentrations were 0.8, 1.5, 2.5, 4.0, and 15.0 μM; UTP concentrations were 20, 40, 70, 150, and 500 μM. Data were fitted as described in the Materials and Methods.

6−12-fold by the inclusion of this concentration of BSA. The effect of the $MgCl_2$ concentration on the initial rate of uridylylation was examined. When UTP was either 20 or 500 μM, the optimal $MgCl_2$ concentration was 25 mM (data not shown). The pH, buffer composition, and buffer concentration were all optimized. Optimal conditions were shown to be Tris-Cl at 100 mM, pH 7.5 (data not shown). The pH optimum was 7.5−8.0. Buffer concentration had little effect within the range 50−150 mM, and above 150 mM, a slight inhibition was observed (data not shown). The rate of reaction in Hepes buffer was slightly less than in Tris-Cl, but the reaction was considerably slower in imidizole buffer (~25% the rate in Tris-Cl, data not shown). Most previous studies of the transferase reaction were performed using methyl-imidazole buffer.

Uridylyl-Removing Assays. The assay was similar to that described previously (*9*). Initial velocities were determined. The inhibition data was fit using the KinetAssyst program. The equation for competitive inhibition was: $V = V_mS/[K_m(1 + I/K_{is}) + S]$ where S is the substrate concentration and I is the inhibitor concentration.

For the Mg^{2+}-UR activity, conditions were 100 mM Tris-Cl, pH 7.5, 100 mM KCl, 50 mM $MgCl_2$, 0.1 mM 2-ketoglutarate (or as indicated), 1 mM DTT, 0.5 mM ATP, 0.3 mg/mL BSA, 1 mM glutamine (or as indicated), and indicated concentrations of proteins. Reactions were started by the addition of PII-[^{32}P]UMP. For the Mn^{2+}-UR activity, conditions were similar except that $MgCl_2$, ATP, and 2-ketoglutarate were omitted (except as indicated), glutamine

was 6 mM, and 1 mM $MnCl_2$ was included.

Deadenylylation of GS−AMP. The adenylylation state of GS was determined by measuring the γ-glutamyl transferase activity of GS in the presence of Mn^{2+} (both forms of GS are active) and in the presence of Mg^{2+} (only unadenylylated GS is active). Deadenylylation was detected as the conversion of GS−AMP to the form that is active in the presence of Mg^{2+}, as described previously (*26*).

Reconstitution of the UTase/UR−PII Monocycle. Reaction conditions were 100 mM Tris-Cl, pH 7.5, 100 mM KCl, 1 mM DTT, 0.5 mM ATP, 0.3 mg/mL BSA, 0.3 mM 2-ketoglutarate, 3 μM PII, 0.3 μM UTase/UR, 0.5 mM [α-^{32}P]UTP, and either 25 mM $MgCl_2$, 1 mM $MnCl_2$, or 25 mM $MgCl_2$ and 1 mM $MnCl_2$, as indicated. The glutamine concentration was varied as indicated, and incubation was at 30 °C for 40 min. Reactions were sampled at various intervals, and the steady-state extent of uridylylation was measured. Examples of typical progress curves are shown in the second paper of three in this issue (*42*).

Thin-Layer Chromatography. Thin-layer chromatography was performed on plastic-backed Cellulose PEI plates (Baker-flex, J. T. Baker, Phillipsburg, NJ). For distinguishing the four different ribonucleoside monophosphates, the buffer was 0.8 M LiCl. To distinguish between ribonucleotide mono-, di-, and triphosphates, the developing buffer was 0.25 or 0.3 M KPi, pH 8.0. As standards, various commercially obtained nucleotides were spotted, as indicated, and detected visually upon short-wave UV illumination. For experiments in which PPi was examined, a small aliquot of commercially obtained [^{32}P]PPi was spotted in adjacent lanes as a marker, and 0.25 mM KPi, pH 8.0, was the solvent. After chromatography, the positions of the standards were visualized using UV illumination and traced using a pencil, and the thin layer plates were subjected to autoradiography. To permit the accurate alignment of the plates with traced spots and the autoradiographs, four small spots were placed on each plate using radioactive ink. Alignment of the autoradiograph with the marked plate allowed for the identification of products.

Binding of 2-Ketoglutarate and ATP to PII and PII−UMP. Binding of effectors to PII and PII−UMP was examined using a filtration method or equilibrium dialysis, as described previously (*10*). Assay conditions were 50 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, and the indicated concentrations of PII or PII−UMP, 2-ketoglutarate, and ATP. [^{14}C]ATP, [^{14}C]-2-ketoglutarate, or α-[^{32}P]ATP were used as radiolabeled ligands, as indicated for each experiment. All assays were performed at 25 °C. Nonspecific binding was determined using reaction mixtures that lacked PII, and these values were subtracted from the raw observed bound fraction to obtain the quantity of specifically bound effector. Binding data was then fit to a model of simple bimolecular receptor−ligand interaction which accounts for ligand depletion: $RL = [(L_t + R_t + K_D) - ((L_t + R_t + K_D)^2 - 4R_tL_t)^{1/2}]/2$; where RL = ligand-PII or ligand-PII−UMP complex, L_t = total ligand; R_t = total PII or PII−UMP, and K_D is the dissociation constant.

RESULTS

The Uridylylation of PII Occurs by a Sequential Reaction Mechanism. The initial rate of the transferase reaction was examined in experiments where each of the substrates was varied. The Lineweaver−Burke transformation from this analysis (Figure 1) revealed a pattern of intersecting lines, indicating a sequential mechanism and eliminating a ping−pong mechanism. We were unable to obtain any evidence for a kinetically competent covalent enzyme intermediate in experiments which focused on the incorporation of label from UTP into the enzyme (data not shown). Thus, if a covalent enzyme intermediate is formed at all, it is extremly transient.

Table 1: Summary of Uridylyltransferase Inhibition Patterns and Constants

inhibitor	var. subs.	fixed subs.	obs. pattern	K_{is} (μM)	K_{ii} (μM)
CTP	UTP	PII (unsat)	C	454.3 ± 44.2	
	PII	UTP (unsat)	UC		594.8 ± 35
PIIY51F	PII	UTP (unsat)	C	2.35 ± 0.3	
	UTP	PII (unsat)	NC	7.41 ± 1.7	15.86 ± 2
PPi	PII	UTP (unsat)	NC	7.18 ± 1.18	35.38 ± 11
	PII	UTP (sat)	UC		113.5 ± 12
	UTP	PII (unsat)	NC	7.80 ± 0.85	124.8 ± 37
	UTP	PII (sat)	NC	19.54 ± 3.64	49.56 ± 9
PII−UMP	PII	UTP (unsat)	C	3.18 ± 0.26	
	PII	UTP (sat)	C	3.75 ± 0.67	
	UTP	PII (unsat)	NC	8.70 ± 2.31	17.14 ± 4
	UTP	PII (sat)	NI[a]		
glutamine	PII[b]	UTP (unsat)	UC		56.2 ± 22
	UTP[b]	PII (unsat)	UC		66.6 ± 4
	UTP[c]	PII (unsat)	NC	112.6 ± 33.4	52.34 ± 3

[a] NI signifies no inhibition. [b] Results from two experiments. [c] Results from a single experiment

Inhibition by Substrate Analogues. CTP and an altered form of the PII protein in which the site of uridylylation has been converted to a nonurdiylylatable residue (Y51F) inhibited the uridylylation of PII by the UTase/UR (Table 1). Inhibition by CTP was competitive with regard to UTP and uncompetitive with regard to PII, and inhibition by the PII−Y51F protein was competitive with regard to PII and noncompetitive with regard to UTP. For both inhibitors, simple linear inhibition was observed (data not shown).

Product Inhibition Studies. Pyrophosphate was a noncompetitive inhibitor with regard to UTP, regardless of whether PII was present in subsaturating or saturating concentration (Table 1). When the inhibition by pyrophosphate was examined with PII as the varied substrate, noncompetitive inhibition was observed when UTP was subsaturating and uncompetitive inhibition was observed when UTP was saturating (Table 1). In all cases, pyrophosphate caused simple linear inhibition (data not shown).

To study inhibition by PII−UMP, we isolated PII−UMP by chromatography after uridylylation as described in Materials and Methods. The PII−UMP obtained was essentially completely uridylylated as indicated by nondenaturing gel electrophoresis (data not shown). When assayed in experiments with labeled UTP, the purified PII−UMP was essentially free of UTase activity (in the presence of excess PII) and had an insignificant concentration of un-uridylylated sites in the presence of excess UTase/UR (i.e., less than 0.2 unmodified sites/PII trimer, data not shown).

PII−UMP was a competitive inhibitor with regard to PII, regardless of the UTP concentration (Table 1). PII−UMP was a noncompetitive inhibitor with regard to UTP, but only when the concentration of PII was limiting. When the concentration of PII was saturating, PII−UMP provided no

Table 2: Kinetic Parameters

Mg^{2+}-UTase		Mg^{2+}-UR	
k_{cat}	137/min	k_{cat}	2.7/min (−gln) 6.5/min (at 2.5 mM gln)
K_m (PII)	3.0 μM		
K_m (UTP)	40 μM	K_D (UMP)	8.4 mM
K_D (PII)	1.8 μM	K_D (PII)	2.0 μM
K_D (PII−UMP)	3.5 μM	K_m (PII−UMP)	2.3 μM (−gln) 1.2 μM (at 1 mM gln) 0.82 μM (at 2.5 mM gln)
K_{act} (2-KG)	5−6 μM (at 0.5 mM ATP)	K_{act} (2-KG)	25−30 μM (±1 mM gln, at 0.5 mM ATP) ~200 μM (−gln, at 30 μM ATP)
K_{act} (ATP)	20 μM (at 33 μM 2-KG) 3 μM (at 2 mM 2-KG)	K_{act} (ATP)	150 μM (±1 mM gln, at 33 μM 2-KG) 45 μM (−gln, at 0.1 mM 2-KG) 5−6 μM (±1 mM gln, at 2 mM 2-KG)
K_i (gln)	70−80 μM	K_{act} (gln)	80 μM
Mn^{2+}-UTase[a]		Mn^{2+}-UR[b]	
k_{cat}	30/min (at 0.1 mM UTP) 22/min (at 3 μM PII)	k_{cat}	61/min (at 6 mM gln) 9.3/min (−gln)
K_m (PII)	1.9 μM	K_m (PII−UMP)	0.32 μM (at 6 mM gln) 0.63 μM (−gln)
K_m (UTP)	20.3 μM	K_D (PII)	0.61 μM
K_{act} (ATP)	20 μM	K_D (UMP)	2.44 μM
K_i (gln)	150 μM	K_{act} (gln)	0.70 mM
Mn^{2+}+ Mg^{2+}-UTase[a]			
k_{cat}	25/min (at 0.1 mM UTP)		
K_m (PII)	2.9 μM		

[a] Measured in the absence of 2-ketoglutarate. [b] Measured in the absence of 2-ketoglutarate and ATP.

inhibition (Table 1). In all cases, the inhibition, when observed, was simple linear inhibition. In combination with the other inhibition patterns (Table 1), this result indicated a mechanism where PII and PII−UMP compete for a site on the free enzyme.

Inhibition by Glutamine. We examined the patterns of inhibition afforded by glutamine. In four experiments, glutamine provided uncompetitive inhibition with regard to either substrate, when the fixed substrate was at unsaturating concentration (Table 1). In another experiment, gradually intersecting lines were obtained (that is, a noncompetitive pattern) when glutamine was varied vs UTP at nonsaturating PII concentration (Table 1). These results suggest that glutamine does not significantly affect the binding of either substrate.

To determine whether glutamine inhibition was due to the inhibition of a post-chemistry step, such as product release, we examined whether a burst of either product was detectable at saturating glutamine concentration and high enzyme concentration. Neither PPi nor PII−UMP demonstrated a burst accumulation in these experiments, indicating that glutamine did not significantly inhibit a step after the chemistry step such as product release (data not shown). Also, we observed that glutamine did not accelerate the reversal of the transferase reaction (pyrophosphorylosis of PII−UMP, Materials and Methods), rather, it inhibited it (data not shown). Together, these results suggest that glutamine affects the rate of the chemistry step.

PII did not demonstrate appreciable substrate inhibition, even in the presence of glutamine (data not shown).

Activation of Uridylyltransferase by ATP and 2-Ketoglutarate. Previous results indicated that the uridylyltransferase activity is activated by 2-ketoglutarate and ATP (*4*−*10*), which bind to PII (*10*). To characterize the 2-ketoglutarate requirement, we essentially repeated the experiments performed previously (*10*) and obtained an apparent K_{act} of 5−6 μM, similar to the reported results (Table 2). We also characterized the binding of 2-ketoglutarate to PII, taking into account the depletion of ligand in the binding assays (Materials and Methods). This analysis resulted in an apparent K_D for 2-ketoglutarate of 7−8 μM (Table 3). Since the K_D and K_{act} for 2-ketoglutarate were nearly identical, this effector may work exclusively by binding to PII.

Table 3: Binding of ATP and 2-Ketoglutarate to PII and PII−UMP[a]

	[PII] (μM)	[2-ketoglutarate]	K_D (μM)	n[b]
	Binding of ATP to PII			
1[c]	1.33	2 mM	2.38 ± 0.01	1.01± 0.00
2	1.33	2 mM	2.00 ± 0.8	0.94 ± 0.07
3[d]	10.0	2 mM	5.40 ± 0.1	0.86 ± 0.00
4	2	33 μM	26.4 ± 5.2	1.04 ± 0.10
5[d,e]	10	33 μM	34.7 ± 2.9	0.68 ± 0.00

	[PII] (μM)	[ATP] (mM)	[NRII] (μM)	K_D (μM)	n[b]
	Binding of 2-Ketoglutarate to PII				
6[e]	1.33	2	−	7.80 ± 0.4	0.38 ± 0.02
7	1.33	2	5	0.46 ± 0.01	0.29 ± 0.01

	[PII−UMP] (μM)	[2-ketoglutarate] (mM)	K_D (μM)	n[b]
	Binding of ATP to PII−UMP			
8[e]	2	2	4.0 ± 1.7	0.88 ± 0.21

	[PII−UMP] (μM)	[ATP] (mM)	K_D (μM)	n[b]
	Binding of 2-Ketoglutarate to PII−UMP			
9[e]	2	2	24.9 ± 4.4	0.83 ± 0.03

[a] Experiments were performed using ^{14}C-labeled ligands and the ultrafiltration method (*10*), except as indicated. Each experiment was performed using seven or eight different ligand concentrations. [b] The capacity/PII or PII−UMP subunit. [c] This experiment was performed using the equilibrium dialysis method (*10*). [d] This experiment was performed using α-[^{32}P]ATP. [e] These values are the mean ± SD from two independent experiments.

To examine the ATP requirement for activation, we first examined whether our purified PII preparation contained ATPase activity, by incubating PII with labeled ATP and subjecting the mixture to analysis by thin-layer chromatography. Although our PII preparation appears quite pure by SDS−gel electrophoresis (not shown), this preparation was

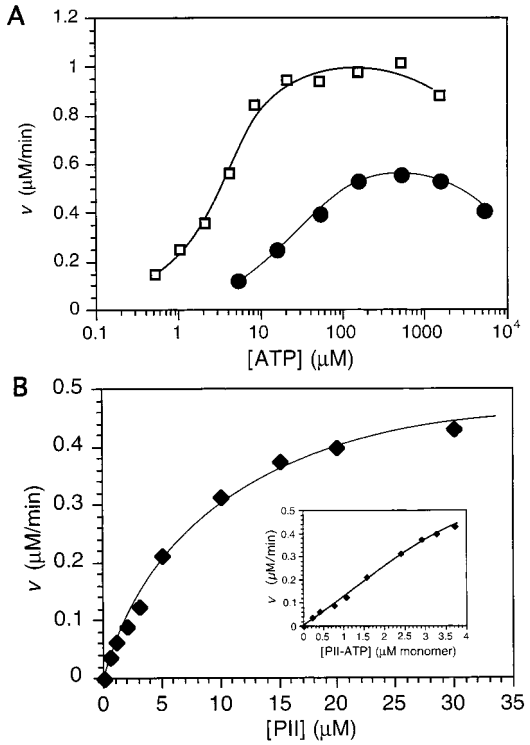

FIGURE 2: Activation of uridylyltransferase by ATP. The reaction conditions were as described in the Materials and Methods. In panel A, PII was 3 μM, UTP was 0.1 mM, and UTase/UR was 0.01 μM. 2-Ketoglutarate concentrations were 33 μM (filled circles) or 2 mM (unfilled squares). In the absence of ATP, the V_o was 0.002 μM/min when 2-ketoglutarate was 33 μM and 0.009 μM/min when 2-ketoglutarate was 2 mM. In panel B, ATP was 5 μM, PII was varied as indicated, and the other conditions were as in panel A. The inset indicates the calculated concentrations of liganded PII subunits, assuming a K_D under these conditions of 30 μM.

found to contain trace ATPase activity, as was the PII preparation used previously to demonstrate that PII bound ATP (*10*, data not shown). This ATPase activity was observed to be separated from PII by gel filtration on Sephadex G-75, and was partially separated from PII by chromatography on Mono-Q, demonstrating that the ATPase activity is not due to PII itself (data not shown). PII protein purified free of ATPase activity by chromatography on both MonoQ and Sephadex G-75 was used in experiments to characterize the ATP requirements for activation and to characterize the binding of ATP to PII (Table 3). The binding of ATP to PII was strongly influenced by the 2-ketoglutarate concentration (Table 3), as noted earlier (*10*); at the saturating 2-ketoglutarate concentration of 2 mM, the apparent K_D for ATP was 2−5 μM, with the lower estimates obtained from experiments where [^{14}C]ATP was the radiolabeled ligand (Table 3). When 2-ketoglutarate was 33 μM, as in our standard transferase assay, an apparent K_D for ATP of ∼30 μM was obtained (Table 3). As shown in Figure 2A, the apparent K_{act} for ATP in the transferase assay was 3 μM when 2-ketoglutarate was 2 mM and was 20 μM when 2-ketoglutarate was 33 μM (Table 2). Since these K_{act}s are nearly identical to the corresponding K_D and the synergy between ATP and 2-ketoglutarate in binding to PII was paralleled in their behavior as activators, these results suggest that ATP activates the uridylyltransferase activity solely by binding the PII protein.

Interestingly, our ligand-binding studies suggest that the trimeric PII protein binds three molecules of ATP and a single molecule of 2-ketoglutarate (Table 3). The presence of the PII-receptor NRII lowered the apparent K_D for 2-ketoglutarate ∼15-fold, without increasing the capacity of PII (Table 3). In contrast, highly purified PII−UMP, essentially free of unmodified PII, is also trimeric and appeared to bind a molecule of ATP and 2-ketoglutarate per subunit (Table 3). This result suggests that there are three binding sites for 2-ketoglutarate in the PII homotrimer and that the binding of 2-ketoglutarate to PII displays negative cooperativity. Also, the K_D for 2-ketoglutarate was different, but the K_D for ATP was essentially the same when PII−UMP was examined (Table 3).

As an additional test of the mechanism of activation by ATP, we examined the initial rate of the transferase reaction in a series of experiments at fixed low ATP (5 μM) and various concentrations of PII. As shown (Figure 2B), PII at 6-fold excess (18-fold PII subunits excess) did not inhibit the reaction, consistent with a model where there is no essential ATP site on the enzyme and the unliganded form of PII does not bind to the enzyme.

Finally, we performed another series of experiments to examine the relationship between PII and ATP, as discussed in Segel (ref *27*, pp 264−267). In one experiment, PII and ATP were combined at identical concentration and simultaneously varied. In a second experiment, PII was varied in fixed large excess over ATP. In a third experiment, ATP was varied in large fixed excess over PII. In the first experiment, reaction rate increased sigmoidally with increasing concentration, while in the second and third experiments, a hyperbolic increase was observed with increasing concentration, and the rate was greater than in the first experiment (data not shown). These results suggest that only the complex of PII and ATP binds to the enzyme.

In a separate experiment, the activation of the UTase activity by ATP and dATP at a concentration of 500 μM was compared; in this experiment, the initial rate of uridylylation activated by dATP was 71% of that seen with ATP (data not shown). None of the other nucleotides (CTP, GTP, UTP) nor dCTP, dGTP, or dTTP were able to activate the UTase activity.

Reversibility of the UTase Reaction. We examined the reversibility of the uridylyltransferase reaction by incubating labeled PII−UMP with enzyme at various reaction conditions and subjecting aliquots of the reaction mixtures to analysis by thin-layer chromatography (data not shown). The UTase/UR catalyzed the hydrolysis of PII−UMP, yielding UMP, in the presence of Mg^{2+}, 2-ketoglutarate, and ATP, as described previously (*6*). This activity (Mg^{2+}-UR activity) required 2-ketoglutarate and ATP as described previously (*6*), and did not require pyrophosphate. Similarly, in the presence of Mn^{2+}, the enzyme catalyzed the hydrolysis of PII−UMP, yielding UMP, in a reaction that did not require ATP and 2-ketoglutarate, as described previously (*6*); this activity (Mn^{2+}-UR activity) also did not require pyrophosphate. In the presence of Mg^{2+}, pyrophosphate, ATP, and 2-ketoglutarate, the enzyme catalyzed the formation of both UMP and UTP from PII−UMP. The UTP formed is apparently due to the reversal of the uridylyltransferase activity. Results from this experiment and additional experiments in which the initial velocity of the reverse of the

Table 4: Summary of Constants for NTPs as Substrates of the Transferase Activity of the UTase/UR[a]

	K_m (mM)	k_{cat} (min^{-1})
UTP	0.04	87.5 (at 4 μM PII)
CTP	0.38	64.5 (at 5 μM PII)
ATP	1.870	29.5 (at 5 μM PII)
GTP	0.94	19.8 (at 5 μM PII)

[a] For reactions with UTP, CTP, and GTP, 0.5 mM ATP was included to activae the transferase reaction. For all reactions, α-[^{32}P] labels were on the nucleotide measured. At least five different nucleotide concentrations were used for each K_m determination.

transferase reaction was measured (not shown) indicated that rate of the reversal of the transferase reaction (k_{cat} ~2/min) was similar to the basal UR activity (k_{cat} ~2.7/min) under these conditions.

Nucleotide Specificity of the Transferase Reaction. To examine this issue, we first measured the inhibition of the rate of uridylylation by other nucleotides in experiments where the label was on UTP, as already noted above for CTP. We observed that ATP at very high concentration was slightly inhibitory (Figure 2A), as was GTP (data not shown). Next, we directly examined the ability of ATP, CTP, and GTP to serve as substrates for the uridylyltransferase and uridylyl-removing (Mn^{2+}-UR) activities, using native gel electrophoresis analysis to detect the formation of modified PII and its hydrolysis (Materials and Methods). Modified PII was detected in the presence of ATP or dATP as the sole nucleotide, but not in the presence of any other nucleotide or deoxynucleotide (including UTP) when these were present as the sole nucleotide (data not shown). When ATP was present, each of CTP, GTP, dTTP, dCTP, dATP, and UTP could serve as substrate for the modification of PII, and each of the modified forms of PII could be hydrolyzed by the Mn^{2+}-UR activity (data not shown).

We measured the apparent K_m for ATP, CTP, and GTP in experiments where the label was on the α-position of these nucleotides. Label from α-[^{32}P]ATP, -CTP, and -GTP was incorporated into PII, in experiments where ATP was present to serve as activator. The kinetic constants resulting from these experiments are summarized in Table 4. In separate experiments, we observed that PII−AMP, PII−CMP, and PII−GMP formation was inhibited by glutamine at concentrations identical to that needed to inhibit the uridylyltransferase activity and that these forms of PII−NMP were readily hydrolyzed by the Mn^{2+}-UR activity of the enzyme in the presence of glutamine and Mn^{2+} (data not shown). In Figure 3, we show the results of experiments in which ATP, CTP, GTP, and UTP were present simultaneously at approximately their in vivo concentration. As shown, when the steady state was obtained under these conditions, 94.1% of the product was PII−UMP, 3% of the product was PII−AMP, 1.7% of the product was PII−CMP, and 1.2% of the product was PII−GMP.

To ensure that the apparent incorporation of AMP, CMP, and GMP into PII was not due to contamination of the commercially obtained nucleotide triphosphates with UTP, we characterized the product of hydrolysis reactions catalyzed by the Mn^{2+}-UR activity on the putative PII−AMP, PII−CMP, and PII−GMP by thin layer analysis (Materials and Methods). This analysis of the products of the Mn^{2+}-UR activity indicated that AMP, CMP, and GMP had indeed been incorporated into PII (data not shown).

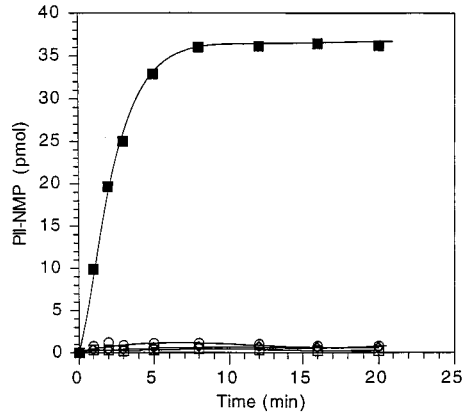

FIGURE 3: Substrate specificity of the UTase reaction. Nucleotidylylation of PII in a reaction mixture containing all four ribonucleotides at their in vivo concentrations. All reaction mixtures contained ATP at 3000 μM, GTP at 900 μM, CTP at 500 μM and UTP at 900 μM. Four different reaction mixtures, each containing identical concentrations of reactants but with a different labeled nucleotide as indicated, were examined. Filled squares, labeled UTP; diamonds, labeled CTP; unfilled squares, labeled GTP; unfilled circles, labeled ATP.

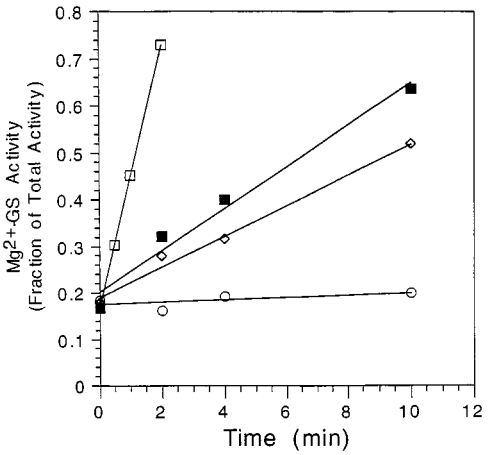

FIGURE 4: Activation of the GS−AMP deadenylylation activity of ATase by PII−UMP, PII−CMP, and PII−AMP. Deadenylylation reaction mixtures included 50 mM Tris-Cl, pH 7.5, 0.1 mM 2-ketoglutarate, 1 mM $MnCl_2$, 10 mM KPi, 0.1 mM ATP, 0.1 μM ATase, 1.8 μM GS−AMP (subunit concentration), and 2.5 μM PII−NMP. After incubating at 30 °C for the indicated times, the fraction of unmodified GS was determined by γ-glutamyltransferase assay in the presence of Mn^{2+} (both forms of GS active) and Mg^{2+} (only unmodified GS is active). Initial rates of deadenylylation were calculated by linear regression using the ENZFIT program. Note that the starting GS population was only about 80% adenylylated. The symbols are open circle, control; open square, PII−UMP; filled square, PII−CMP; diamond, PII−AMP.

Activation of the Deadenylylation Activity of ATase by PII−AMP, PII−CMP, and PII−UMP. Previous results indicated that PII−UMP is an essential activator for the deadenylylation activity of the ATase. We examined whether PII−AMP and PII−CMP could replace PII−UMP in activating the ATase (Figure 4). As shown, PII−CMP and PII−AMP could activate the deadenylylation of GS by the ATase, but not as well as PII−UMP. We also examined whether the modification of PII with AMP or CMP prevented the activation of the phosphatase activity of NRII by PII. The results of these experiments indicated that, like PII−UMP, PII−AMP and PII−CMP did not activate the NRII

Table 5: Inhibition of the Mg^{2+}-UR and Mn^{2+}-UR by Nucleotides and PII

Mg^{2+}-UR		Mn^{2+}-UR	
addition	relative V_o (%)	addition	relative V_o (%)
control (+gln)	100	control (+gln)	100
−gln	24.1	0.5 μM PII	73
6 μM PII	39.6	1.5 μM PII	55
0.15 mM UMP	86.4	4 μM PII	20
0.15 mM CMP	81.5	1.5 μM UMP	64
3 mM ATP	113.4	0.1 mM ATP	65
1 mM GTP	91.7	2 mM ATP	8
1 mM CTP	90.2	0.1 mM UTP	67
1 mM UTP	66.5	2 mM UTP	7
		0.1 mM CTP	75
		2 mM CTP	12
		0.1 mM GTP	60
		2 mM GTP	8
		1.5 μM UMP + 0.5 μM PII	58

phosphatase activity (data not shown).

Characterization of the UR Activities and the Effect of Metal Ions on the Uridylyltransferase and Uridylyl-Removing Activities. As described previously (*6*, *7*), the UTase/UR enzyme has two uridylyl-removing activities: in the presence of Mn^{2+}, the enzyme rapidly cleaves the substrate PII−UMP, forming UMP and PII. This activity was activated by glutamine, and did not require 2-ketoglutarate or ATP, as expected (Table 2). In the presence of Mg^{2+}, uridylyl-removing activity was only observed in the presence of 2-ketoglutarate and ATP, as noted previously (*6*, *7*). The Mg^{2+}-supported activity was also activated by glutamine (Table 2). We shall refer to these activities as the Mn^{2+}-UR and Mg^{2+}-UR, respectively. In addition to the uridylyl-transferase activity in the presence of Mg^{2+}, the enzyme displayed transferase activity in the presence of Mn^{2+} (refs *6* and *7*; Table 2), which we shall refer to as the Mn^{2+}-UTase.

Characterization of the Mg^{2+}-UR. The Mg^{2+}-UR activity required 2-ketoglutarate and ATP (refs *6* and *7*; this work), and was activated about 4-fold by glutamine, with an apparent K_{act} of 80 μM (Table 2). The optimal Mg^{2+} concentration was 50 mM (data not shown). Under optimal conditions with saturating glutamine (2.5 mM), the k_{cat} was 6.5 min^{-1} (Table 2), with a K_m for the substrate PII−UMP of 0.82 μM (Table 2).

The Mg^{2+}-UR was inhibited by both PII and UMP, but the inhibition by UMP was considerably less effective (~3000-fold) than that seen with the Mn^{2+}-UR (Tables 2 and 5). When the saturating concentration of 1 mM glutamine was present, inhibition by UMP occurred with an apparent K_i of 8.4 mM (Table 2), similar to earlier results (*6*, *7*). In experiments where substrate was varied at fixed product concentrations, inhibition by either product was purely competitive, consistent with a mechanism involving random release of products and rapid equilibrium substrate binding. The Mg^{2+}-UR was not strongly inhibited by nucleoside triphosphates or by CMP (Table 5).

Activation by ATP was strongly dependent on the 2-ketoglutarate concentration. At the saturating 2-ketoglutarate concentration of 2 mM, the K_{act} for ATP was 5−6 μM (Table 2) and the K_D for dissociation of ATP from PII−UMP was 4 μM (Table 3), suggesting that ATP works by binding to the substrate, PII−UMP. When 2-ketoglutarate was 100 μM, the K_{act} for ATP was 45 μM, and when 2-ketoglutarate was 33 μM, the K_{act} for ATP was 150 μM. Similar binding studies in the presence of lower concentrations of 2-ketoglutarate were not practical, owing to the higher apparent K_D for ATP. The K_{act} for ATP was not altered by the presence of glutamine (Table 2).

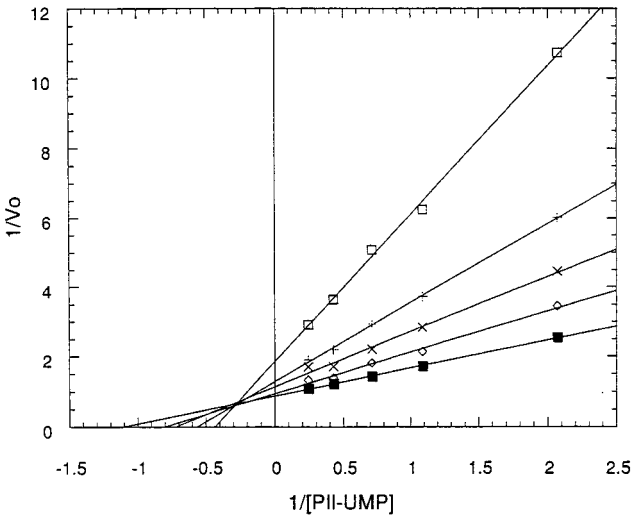

FIGURE 5: Activation of the Mg^{2+}-UR by glutamine. The reaction conditions were as in the Materials and Methods, except that 2-ketoglutarate was 1 mM and the enzyme was 0.2 μM. Glutamine concentrations were (from lower curves to upper) 2.5, 0.15, 0.075, 0.035, and 0 mM. The double reciprocal transformation is shown. Factors α and β, defined as the effect of activator on K_m and rate constant, respectively, from this experiment and another experiment were 0.39 ± 0.07 and 2.28 ± 0.22, respectively (see ref *27*).

At saturating ATP (0.5 mM), the K_{act} for 2-ketoglutarate was 25−30 μM, whether glutamine was present or not (Table 2). However, when ATP was 30 μM, the apparent K_{act} for 2-ketoglutarate was 200 μM (Table 2). Thus, the synergy between 2-ketoglutarate and ATP was again observed, and since the 2-ketoglutarate K_D and K_{act} values at saturating ATP were similar, it is likely that 2-ketoglutarate also acts by binding to the PII−UMP substrate.

To further investigate the relationship between glutamine and 2-ketoglutarate, each of these activators was varied at four different concentrations of the other and the effect on the initial rate of the UR reaction was determined. The UR activity did not seem to be regulated by the ratio of glutamine to 2-ketoglutarate (data not shown), rather, the activity seemed to be independently activated by these two effectors.

Glutamine exhibited nonessential mixed-type activation, both lowering the apparent K_m for PII−UMP and increasing the rate of catalysis (Figure 5). This pattern of activation is consistent with models where glutamine may bind either to both the free enzyme and the ES complex or to the ES complex alone.

Since the Mg^{2+}-UR activity was not robust (k_{cat} 6.5 min^{-1} with saturating glutamine), we considered the possibility that the activity we were observing was a basal activity that could be further stimulated by a small molecule activator. We examined [or reexamined (*6*, *7*)] the effect of the following compounds: ammonium chloride, glutamate, cAMP, coenzyme A, acetyl-CoA, fructose-1,6-diphosphate, serine, glucosamine-6-phosphate, phospho*enol*pyruvate, tryptophan, carbamyl-phosphate, AMP, ADP, alanine, glycine, pyrophosphate, inorganic phosphate, methionine, 3-phosphoglyc-

erate, acetyl−phosphate, and pyruvate. None of these compounds, at 1 mM concentration, provided any activation of the Mg^{2+}-UR activity (data not shown), and inorganic phosphate at 10 mM provided no activation. Furthermore, we examined whether purified GS, GS−AMP, adenyltransferase, NRI, NRII, or the combination of GS, glutamate, ammonium chloride, and ATP could activate the Mg^{2+}-UR activity, but none of these was stimulatory (data not shown). Under the latter conditions, we observed that the UR rate was decreased, but that this could be overcome by the inclusion of a high concentration of ATP (data not shown). This is not unexpected, because the GS biosynthetic reaction consumes ATP.

Characterization of the Mn^{2+}-UR Activity. As noted earlier, the Mn^{2+}-UR activity is considerably more robust than the Mg^{2+}-UR ($k_{cat} = 61$ min^{-1} vs $k_{cat} = 6.5$ min^{-1}, Table 2). At 1 mM Mn^{2+} and saturating 6 mM glutamine, the K_m for PII−UMP was lower than seen with the Mg^{2+}-UR (0.32 μM vs 0.82 μM, Table 2). Both PII and UMP were strongly inhibitory (Table 5), and both products demonstrated competitive inhibition, consistent with a mechanism involving random product release from the enzyme and rapid equilibrium of substrate binding. That is, both UR activities occur by the same kinetic mechanism. Inhibition by the combination of both products was additive or nearly so. For example, concentrations of PII (0.5 μM) and UMP (1.5 μM) that provided 27 and 36% inhibition, respectively, in combination provided 42% inhibition of the initial rate. The activity was stimulated approximately 7-fold by glutamine, with an apparent K_{act} of 0.7 mM (Table 2), similar to our earlier estimate of 1.0 mM (*10*).

As shown above and previously (*6*, *7*), the Mn^{2+}-UR was strongly inhibited by UMP; past results show that it was also strongly inhibited by CMP (*6*, *7*). This activity was also strongly inhibited by nucleoside triphosphates (Table 5). Some of this inhibition is likely due to chelation of the Mn^{2+} ions by the nucleoside triphosphates at high concentration.

Unlike the Mg^{2+}-UR, the Mn^{2+}-UR did not require ATP and 2-ketoglutarate as essential activators. Each of these effectors, when alone, did not provide significant inhibition of the Mn^{2+}-UR, but the combination of 0.2 mM ATP and 1 mM 2-ketoglutarate provided ~44% inhibition of the initial rate (data not shown). Since 2-ketoglutarate and ATP synergistically bind the substrate, PII−UMP, obviously the interaction of the substrate with the enzyme was altered in the presence of Mn^{2+} ions.

Characterization of the Mn^{2+}-UTase. We examined the effect of the presence of Mn^{2+}, either as the sole metal or in combination with Mg^{2+}, on the uridylylation of PII. A comparison of the rates of uridylylation of PII in the presence of Mg^{2+} (25 mM), Mn^{2+} (1 mM), or the combination of Mn^{2+} (1 mM) and Mg^{2+} (10 mM) is shown in Table 2. The UTase activity was most robust in the presence of Mg^{2+} alone; the rate in the presence of both metals was similar to that obtained with Mn^{2+} alone.

Like the Mg^{2+}-UTase, the Mn^{2+}-UTase required ATP as an essential activator, at similar concentration ($K_{act} = 20$ μM when 2-ketoglutarate was absent, Table 2). Also, like the Mg^{2+}-UTase, the Mn^{2+}-UTase had low substrate specificity and could, for example, use ATP in place of UTP, albeit with an elevated K_m (data not shown). However, several

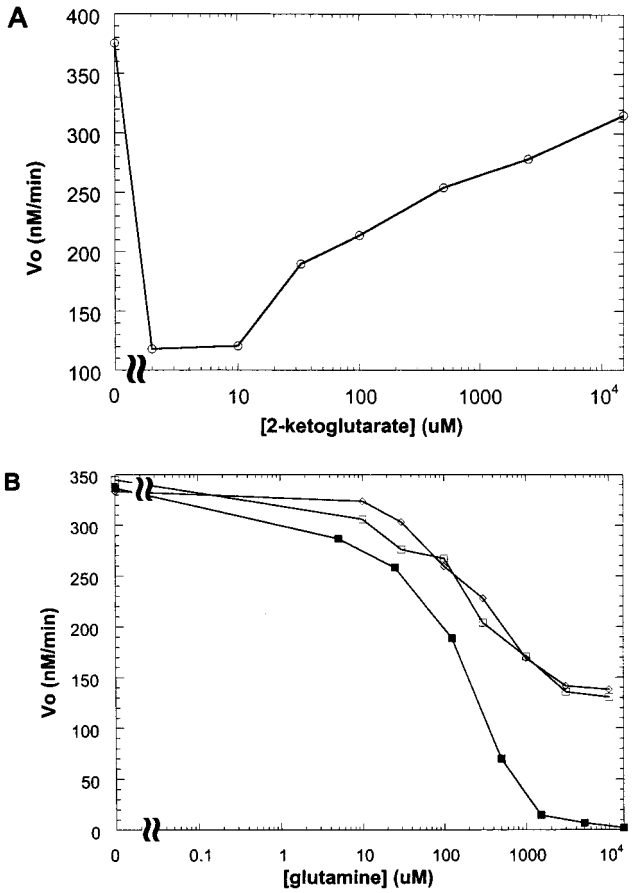

FIGURE 6: Inhibition of the Mn^{2+}-UTase activity by 2-ketoglutarate and glutamine. The Mn^{2+}-UTase activity was measured as described in the Materials and Methods, except that the 2-ketoglutarate and glutamine concentrations were varied as indicated. (A) The effect of 2-ketoglutarate in the absence of glutamine. (B) The effect of glutamine in the absence of 2-ketoglutarate (filled square), in the presence of 33 μM 2-ketoglutarate (unfilled diamond) or 15 mM 2-ketoglutarate (unfilled square). These curves were the results from separate experiments, using different concentrations of the enzyme.

other properties of the Mn^{2+}-UTase differ considerably from the Mg^{2+}-UTase, as described below.

The most striking difference between the Mg^{2+}-UTase and the Mn^{2+}-UTase was that the former required 2-ketoglutarate as an essential activator, whereas the latter activity did not require 2-ketoglutarate and was strongly inhibited by low concentrations of this compound (Figure 6A). The pattern of inhibition by 2-ketoglutarate was curious; at very low 2-ketoglutarate concentration, the activity was inhibited ~60%, but at higher concentrations of 2-ketoglutarate, the inhibition was partially relieved (Figure 6A). Also in contrast to the Mg^{2+}-UTase, the Mn^{2+}-UTase activity was only partially (~60%) inhibited by glutamine in the presence of 2-ketoglutarate (Figure 6B). In the absence of 2-ketoglutarate, the Mn^{2+}-UTase was completely inhibited by glutamine, with a K_i of ~150 μM (Figure 6B). In the presence of 2-ketoglutarate, the K_i for the partial inhibition by glutamine was ~150 μM at both high and low 2-ketoglutarate concentrations.

The effector 2-ketoglutarate binds the PII protein, and was not observed to bind to the UTase/UR (*10*). Thus, it is difficult to compare the K_m for PII for the Mg^{2+}-UTase (3 μM), measured in the presence of 2-ketoglutarate, with the K_m for PII for the Mn^{2+}-UTase (1.9 μM, Table 2), measured

Table 6: Comparison of the Initial Rates of Uridylylation of Mutant PII Proteins in the Presence of Mg^{2+} Alone or the Combination of Mg^{2+} and Mn^{2+}

PII protein	relative V_o (Mg^{2+})	relative V_o (Mg^{2+} + Mn^{2+})
wild-type	1[a]	1[b]
T29M	0.06	0.51
G37A	0.28	0.29
R38H	0.40	2.46
Q39E	0.03	0.70
K40N	0.28	1.04
G41A	0.15	4.08
Y46F	0.38	1.33
A49P	0.88	0.96
E50Q	0.07	1.91
V53G	0.16	2.50
R79H	0.80	0.87
T83N	0.15	0.93
G84A	0.75	1.01
K90R	0.14	0.33

[a] 2.55 μM/ min, 0.066 μM UTase/UR. [b] 8.32 μM/min, 1.6 μM UTase/UR. When the same amount of enzyme is used, the V_o is about 7.4-fold higher in Mg^{2+} when wild-type PII was examined.

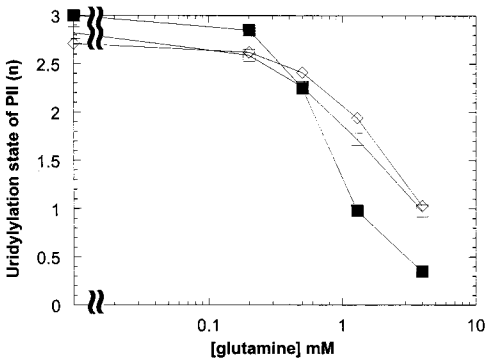

FIGURE 7: Reconstitution of the UTase/UR−PII monocycle. Reaction mixtures were as in the Materials and Methods and contained either 25 mM $MgCl_2$ (filled squares), 1 mM $MnCl_2$ (unfilled squares), or the combination of 25 mM $MgCl_2$ and 1 mM $MnCl_2$ (unfilled diamonds). Reaction mixtures were incubated at 30 °C, and the steady-state level of PII uridylylation (*n*) was determined. Since PII is trimeric, *n* may vary from 0 to 3.

in the absence of 2-ketoglutarate. Nevertheless, it is clear that the interaction of PII with the enzyme is considerably different in the presence of these two different metals. We examined the uridylylation of 14 mutant PII proteins (*22*) in the presence of Mg^{2+} alone, and in the presence of both Mg^{2+} and Mn^{2+}. There was a remarkable difference in the rate of uridylylation of certain altered PII proteins. In particular, the uridylylation of PII proteins containing the T29M, Q39E, K40N, G41A, and E50Q alterations was most severely affected (Table 6). This result further suggests that the binding of PII to the enzyme was altered by the presence of Mn^{2+} metal ions.

Reconstitution of the UTase/UR−PII Monocycle. We examined the regulation of PII uridylylation state by small molecule effectors in reaction mixtures that initially contained PII, the UTase/UR, UTP, and various effector concentrations. The behavior of the reconstituted system containing Mg^{2+} as the sole metal ion is described in the second of three papers in this issue (*42*). In these reaction mixtures, PII uridylylation rapidly reached a steady state that was characteristic of the glutamine concentrations (Figure 7, also see ref *42*). We also examined the regulation of PII uridylylation state by glutamine in reaction mixtures containing Mn^{2+} as the sole metal ion, and containing both Mg^{2+} and Mn^{2+}. A reconstituted system containing both Mg^{2+} and Mn^{2+} metal ions was described previously (*3*). In our experiments, only the reconstituted system containing Mg^{2+} as the sole metal ion responded efficiently to glutamine when this effector was varied through its physiological range (Figure 7). These results show that the Mg^{2+}-UR activity is sufficient to balance the Mg^{2+}-UTase activity.

DISCUSSION

Hypothesis for the Role of Small Molecules in the Regulation of the UTase/UR−PII Monocycle and Nitrogen Assimilation in Vivo. In intact cells, both the regulation of glutamine synthetase adenylylation state and the regulation of transcription of the Ntr regulon appear to be due to signals of nitrogen status and signals of carbon status. Earlier work had suggested that the key signal molecules were 2-ketoglutarate, a signal of carbon sufficiency, and glutamine, a signal of nitrogen sufficiency (*4*−*8*, *28*). In intact cells, the carbon and nitrogen signals appear to act antagonistically. One part of this process is the regulation of PII uridylylation state, which, in turn, is due to the UTase and UR activities studied here. Our analysis suggests that, at physiological conditions, PII uridylylation state is regulated mainly by the glutamine concentration. Thus, we hypothesize that the UTase/UR−PII monocycle serves as a glutamine-sensing apparatus. This, in turn, suggests that the carbon signals regulating nitrogen assimilation, and in particular 2-ketoglutarate, act at another point in the signal transduction process.

PII and PII−UMP are the sensors of the 2-ketoglutarate signal. Our binding studies showed that PII trimers bound a single molecule of this effector, while PII−UMP trimers bound three molecules. Thus, the binding of a molecule of 2-ketoglutartate to PII may result in strong negative cooperativity for the binding of additional molecules. The methods used in our binding studies could only reliably detect dissociation constants similar to the PII concentration. However, our kinetic data from the study of 2-ketoglutarate inhibition of the Mn^{2+}-UTase (Figure 6A) strongly suggested that 2-ketoglutarate binding cannot be explained by independent and identical 2-ketoglutarate binding sites on the PII trimer. We hypothesize that PII may exist in two conformations, depending on whether a single molecule or more than one molecule of 2-ketoglutarate is bound. The former of these is apparently unable to interact with the Mn^{2+}-UTase, while the latter can (Figure 6A). These results are consistent with earlier data showing that the interaction of PII with NRII was stimulated by a low concentration of 2-ketoglutarate, but inhibited by a higher concentration of 2-ketoglutarate (*10*, *30*). That is, the interaction of PII with NRII was regulated by 2-ketoglutarate in the absence of the UTase/UR (*10*, *30*). The dissociation constant for the first molecule of 2-ketoglutarate to bind PII (Table 3) is well below the in vivo range [0.1−0.9 mM (*28*)]. The dissociation constant for the additional 2-ketoglutarate molecules, estimated as 200 μM from previous data and Figure 6A, is within the physiological range. Our data suggest that the UTase/UR, in the presence of Mg^{2+}, can interact with either conformation of PII equally well, and we hypothesize that the allosteric regulation of PII conformation by 2-ketoglutarate controls the interaction of PII with its other receptors.

This hypothesis is consistent with the results obtained in the second and third papers of three in this issue (*42*, *43*).

Role of the Small Molecule Effectors ATP, 2-Ketoglutarate, and Glutamine in Regulating the UTase and UR Activities. Our results suggest that 2-ketoglutarate and ATP, which are essential activators of both the Mg^{2+}-UTase and Mg^{2+}-UR activities (*6*, *9*−*11*), act solely by binding to the substrates PII and PII−UMP. Previous results indicated that both ATP and 2-ketoglutarate are bound synergistically with high affinity by the PII protein, and that the concentration of 2-ketoglutarate required to activate the uridylyltransferase activity was similar to the K_D for the dissociation of 2-ketoglutarate from PII in the presence of saturating ATP (*10*, *11*). Kinetic and binding studies did not demonstrate any interaction of these effectors with the enzyme (ref *10*; this study). We showed that the synergism between ATP and 2-ketoglutarate was also evident in the K_{act} for these effectors, for both transferase and (Mg^{2+}) uridylyl-removing activities, and that the K_{act} and K_D at various conditions were similar. Thus, all effects of ATP and 2-ketoglutarate may be explained by the binding of these effectors to PII and PII−UMP. This conclusion was further tested for ATP activation of the transferase, by examining the effect of variation in the PII concentration at low fixed ATP concentration. At these conditions, excess PII was not an effective inhibitor of the transferase activity. Since virtually all of the ATP should have been complexed with PII at the elevated concentrations of PII (Figure 2B), the possibility that there is an essential ATP-site on the enzyme seems to be excluded by the experimental result. Furthermore, PII that was not complexed with ATP was not an effective inhibitor of the reaction. Yet another experiment led to the same conclusion: when ATP and PII were at identical concentrations, the initial rate increased sigmoidally with increasing concentrations. In contrast, a hyperbolic increase and greater rates were obtained when either ATP or PII was in large fixed excess. These results are consistent with the hypothesis that ATP activated the transferase reaction solely by binding to PII.

We previously showed that the Mg^{2+}-UTase activity, which required the essential activator 2-ketoglutarate and was inhibited by glutamine, was regulated independently by these effectors (*10*). That is, the concentration of glutamine required to inhibit the enzyme was the same regardless of the 2-ketoglutarate concentration. Here, we observed that the Mg^{2+}-UR activity is also activated independently by 2-ketoglutarate and glutamine. Considering the reported intracellular concentrations of 2-ketoglutarate [0.1−0.9 mM (*28*)] and ATP [3 mM (*31*)], it seems that the both the UTase activity and UR activities should be mainly regulated by the glutamine concentration in vivo. Furthermore, neither Mg^{2+}-dependent activity was significantly inhibited by high 2-ketoglutarate. Thus, the uridylylation state of PII should be regulated in vivo mainly by the concentration of glutamine.

The mechanism of inhibition of the uridylylation reaction by glutamine and activation of the uridylyl-removing activity by glutamine are not elucidated by the data presented here. However, for the transferase activity the uncompetitive inhibition patterns obtained vs each substrate suggest that glutamine mainly bound the central complex and did not affect the binding of either substrate. This finding is in agreement with the previous reports (*3*, *7*). Since we could not detect a burst of either product in the presence of glutamine in transferase experiments designed to permit the observation of one enzyme equivalent of product, we must conclude that glutamine did not inhibit a post-chemistry step such as release of either product. Also, glutamine was a potent inhibitor of the reversal of the transferase reaction (pyrophosphorolysis of PII−UMP). Furthermore, glutamine did not increase substrate inhibition of the reaction by PII. In the aggregate, these results suggest that glutamine inhibited the transferase activity by binding the central complex and inhibiting the rate of the catalytic step.

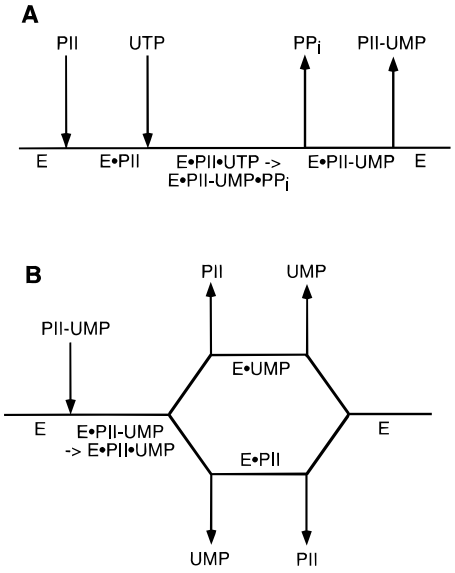

FIGURE 8: Proposed uridylyltransferase and uridylyl-removing reaction schemes depicted in the nomenclature of Cleland. (A) The uridylyltransferase scheme. (B) The uridylyl-removing enzyme scheme.

Studies with the Mg^{2+}-enzyme suggest that the glutamine site responsible for the inhibition of the UTase activity may be identical to the glutamine site responsible for stimulation of the UR activity (Table 2). When Mg^{2+} was the sole metal, both effects of glutamine were seen at similar concentration (70−80 μM). The nonessential mixed-type activation of the Mg^{2+}-UR activity by glutamine may be due to the binding of this effector to both free enzyme and central complex or to only the central complex. If the effects of glutamine are mediated from a single site, the latter could explain the activation. The presence of Mn^{2+} altered the regulation of both UTase and UR activities by glutamine. Several models may account for this; for example, the presence of Mn^{2+} in the enzyme may distort the enzyme, altering communication between the active site and glutamine site.

Kinetic Mechanism of the UTase and UR Reactions. Steady-state kinetic analysis indicated a sequential ordered bi−bi reaction mechanism for the uridylyltransferase reaction and a random release of products for the uridylyl-removing reaction, as depicted using the Cleland nomenclature in Figure 8. These are the only kinetic models supported by the complete set of inhibition patterns (*27*, *29*). The binding order of substrates and order of product release deduced here for the uridylyltransferase activity are similar to previous results obtained with the related enzyme DNA polymerase β of eucaryotic cells (*21*). We were unable to obtain any evidence for a kinectically competent covalent intermediate in the transferase reaction, and the sequential nature of the

reaction mechanism was clearly indicated by the intersecting pattern of lines in the Lineweaver−Burke transformations shown in Figure 1. The requirement for a ternary complex for activity further suggests against a reactive covalent enzyme intermediate. The kinetic constants obtained in our study are reasonable with regard to the likely intracellular concentrations of PII and UTP. It was previously shown that the uridylylation of PII by the UTase/UR was "non-processive", that is, the likelihood of a PII monomer becoming uridylylated was not affected by the uridylylation state of the other monomers within the PII trimer (*11*). That result is consistent with the linear kinetic patterns reported here.

Substrate Specificity of the UTase. We apparently conducted the first study of the nucleotide substrate specificity of the UTase. The nucleotide substrate specificity of the UTase activity was not strict; PII covalently containing AMP, CMP, GMP, dAMP, dCMP, and dTMP could be readily formed, as long as ATP or dATP was present to activate the reaction by binding to PII. Furthermore, the K_m for each of ATP, CTP, and GTP was similar to the in vivo concentrations of these nucleotides (*31*), while the K_m for UTP was ~22.5-fold below the in vivo UTP concentration (*31*). In a reconstruction experiment where all four ribonucleotides were present at their in vivo concentrations, PII−UMP constituted 94% of the modified PII, but the other forms of modified PII were readily detected. Thus, these minor forms of PII may be formed in vivo as well. In that case, it was possible that these minor forms of modified PII could have a role in nitrogen regulation, particularly if they differ from PII−UMP in their interaction with the ATase or NRII. However, that possibility was dimished by our observation that none of the modified PII proteins were able to activate the NRII phosphatase activity, and PII−AMP and PII−CMP were, like PII−UMP, able to activate the deadenylylation activity of ATase, although not as effectively as PII−UMP. Apparently, the interaction of PII−UMP with the adenylyltransferase does not require specific contacts with the uridylyl moiety.

The UTase and UR Activities Probably Occur at a Single Catalytic Center on the Enzyme. In another paper, we show that site-specific mutagenesis of the highly conserved sequence shared with other nucleotidydyltransferases can result in mutations affecting only the UTase activity, the UR activity, or both activities (*40*). Those results strongly suggested that the UTase and UR reactions occur at a single catalytic center on the enzyme. The kinetic data presented here are consistent with this hypothesis. Previous studies of the UTase/UR have mainly focused on the Mn^{2+}-UR activity and the Mg^{2+}-UTase activities, as these are the more robust. When considering these two activities, it is easy to understand how the many differences in regulation by small molecule effectors, inhibition by nucleotides, and differences in the interaction with the PII could give rise to the idea that the two reactions occurred at different catalytic centers on the enzyme (*32*). However, our study suggests that these differences mainly result from the identity of the metal ion, and not from two independent active sites.

The kinetic data are consistent with a single binding site for PII/PII−UMP, which was affected by the identity of the metal ion cofactor (Table 2). For example, in the presence of Mg^{2+} as the sole metal, PII bound to the enzyme with K_D of ~2 μM, when either UTase (1.8 μM) or UR activity (2.0 μM, Table 2, $K_D = K_{is}$ in a rapid equilibrium mechanism) was examined. Also, the K_D for PII−UMP was similar for the Mg^{2+}-UTase (3.5 μM = $K_{is(PII-UMP)}$ in competitive inhibition) and Mg^{2+}-UR [2.3 μM = $K_{m(PII-UMP)}$ in the absence of glutamine, note that since the observed rate is so slow (k_{cat} = 6.5 min^{-1}), $K_m \approx K_D$ in a rapid equilibrium mechanism]. Thus, both UTase and UR activities may utilize a common PII/PII−UMP site. The kinetic data indicate that PII and PII−UMP compete for this site. The only alternative possibility is that the enzyme contains two identical sites for PII/PII−UMP and that occupancy of one of these has no effect on occupancy of the other.

Although the affinity of PII and PII−UMP for the different forms (Mg^{2+}, Mn^{2+}) of the enzyme differ only by severalfold, a dramatic metal-dependent difference in the ability of the UTase activity to accept certain mutant PII proteins as substrate was observed (Table 6), indicating a metal-dependent difference in the PII site. The uridylylation of one of the altered PII proteins examined, E50Q, is of note. In separate experiments with intact cells, this protein behaved as if it was unable to be uridylylated (A.J.N., M. R. Atkinson, and B. R. Schefke, unpublished data), which was not surprising since the *Klebsiella pneumoniae* PII protein containing a mutation at this position was reported earlier to have similar behavior (*33*). In the presence of Mg^{2+}, this PII protein was only uridylylated 7% as well as the wild-type PII (*22*), whereas it was uridylylated about twice as well as the wild-type protein in the presence of both metals (Table 6). Thus, the earlier in vivo results (ref *33*; unpublished data) support the idea that the physiologically relevant activity is the Mg^{2+}-dependent activity. The deficiency in the case of the Mg^{2+}-dependent uridylylation of the E50Q protein seemed to be primarily on the V_{max} of the uridylylation reaction [k_{cat} = 6.2 min^{-1} vs 137 min^{-1} for wild-type PII (*22*)]. The K_m for this protein (3.59 μM) was similar to the K_m for wild-type PII (3.0 μM).

Further illustrating the metal ion effects on the PII site, the Mn^{2+}-dependent UTase activity was inhibited by 2-ketoglutarate, as already noted. Thus, quantitative comparisons of the binding of PII to the two enzyme forms (Mg^{2+}, Mn^{2+}) were confounded, but the interaction is quite different.

The Physiological Metal Ion Cofactor for the UTase/UR Is Probably Mg^{2+}. We observed that the presence of Mn^{2+} had several dramatic effects on the enzyme, in addition to affecting the PII/PII−UMP site. The interaction of the enzyme with nucleotides was clearly affected. In the presence of Mg^{2+} as the sole metal, the UR activity was not appreciably inhibited by ATP, GTP, or CTP, and UTP, UMP, and CMP were weak inhibitors. In contrast, the Mn^{2+}-UR was strongly inhibited by nucleotide triphosphates and, as noted earlier (*6*, *7*), by CMP and UMP. At physiological concentrations of these compounds (*31*), the Mn^{2+}-UR would likely be completely inhibited, as noted over 20 years ago for CMP and UMP (*6*). In addition, the regulation of the both the UTase activity and the UR activity by glutamine was affected by Mn^{2+}. When 2-ketoglutarate was present, the Mn^{2+}-UTase was only partially inhibited by glutamine.

In many cases, the activity of purified enzymes involved in phosphoryl transfers is increased or altered in the presence of Mn^{2+}, in vitro. For example, DNA polymerases may have primer-dependent RNA polymerase activity in the presence

of Mn^{2+} (*34*). Also, the fidelity of DNA replication is affected by the presence of Mn^{2+} (*35*−*37*), and this property forms the basis for a common in vitro mutagenesis technique (*38*). Since the active site of the UTase/UR is homologous to DNA polymerase β, and the kinetic mechanism is similar to this DNA polymerase, it is reasonable to suggest that the Mn^{2+}-dependent activities of the UTase/UR are likewise of no physiological significance.

We studied the regulation of the steady-state level of PII uridylylation in reconstituted systems containing the UTase/UR, PII, UTP, 2-ketoglutarate, ATP, and either Mg^{2+}, Mn^{2+}, or the combination of Mg^{2+} and Mn^{2+}. A reconstituted system with both metals was reported previously (*3*, *39*). In our experiments, only the reconstituted system containing Mg^{2+} as the sole metal ion responded efficiently to physiological concentrations of glutamine (*41*). In this reconstituted system, PII uridylylation state varied from nearly complete uridylylation ($n = 3$) in the absence of glutamine to almost complete deuridylylation ($n = 0.35$) at 4 mM glutamine. Furthermore, the sharpest regulation by glutamine was observed when this was between 0.2 and 1 mM. These concentrations correspond well to recent measurements of the glutamine concentration in cells under various conditions (*41*), suggesting that Mg^{2+} is the physiologically important metal ion cofactor. In contrast, in our reconstituted systems containing Mn^{2+}, PII was uridylylated to a greater extent (Figure 7). This may be due to the fact that inhibition of the Mn^{2+}-Utase activity by glutamine was only partial (Figure 6B).

Authors note: the full set of kinetic plots and thin layer chromatograms resulting from this work are available from the corresponding author.

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