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The Robustness of the *Escherichia coli* Signal-Transducing UTase/UR-PII Covalent Modification Cycle to Variation in the PII Concentration Requires Very Strong Inhibition of the UTase Activity of UTase/UR by Glutamine

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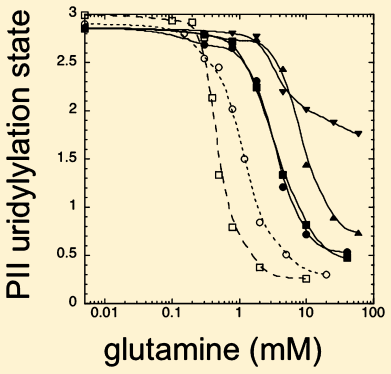
Supporting Information

ABSTRACT: Uridylyltransferase/uridylyl-removing enzyme (UTase/UR) catalyzes uridylylation of PII and deuridylylation of PII-UMP, with both activities regulated by glutamine. In a reconstituted UTase/UR-PII cycle containing wild-type UTase/UR, the steady-state modification of PII varied from nearly complete modification to nearly complete demodification as glutamine was varied, whether the level of PII was saturating or unsaturating, but when a His-tagged version of UTase/UR was used, the robustness to variations in PII concentration was lost and the range of PII modification states in response to glutamine became smaller as the PII concentration increased. The presence of the His tag on UTase/UR did not alter PII substrate inhibition of the UT activity and had little effect on the level of the UT activity but resulted in a slight defect in UR activity. Importantly, at high PII concentrations, glutamine inhibition of the UT activity was incomplete. We hypothesized that binding of PII to the UR active site in the HD domain was responsible for PII substrate inhibition of the UT activity and, in the His-tagged enzyme, also weakened glutamine inhibition of the UT activity. Consistent with this, three different UTase/UR proteins with HD domain alterations lacked substrate inhibition of UT activity by PII; in one case, the HD alteration eliminated glutamine regulation of UT activity, while for the other two proteins, alterations of the HD domain partially compensated for the effect of the His tag in restoring glutamine regulation of UT activity. We conclude that very strong inhibition of UT activity was required for the UTase/UR-PII cycle to display robustness to the PII concentration, that in the wild-type enzyme PII brings about substrate inhibition of the UT activity by binding to the HD domain of the enzyme, and that addition of an N-terminal His tag resulted in an altered enzyme with subtle changes in the interactions between domains such that binding of PII to the HD domain interfered with glutamine regulation of the UT domain.



Biological signal transduction systems must produce an accurate output signal in response to an input stimulation in the heterogeneous and stochastic environment of the cell, where variations in the concentrations of proteins and small molecules that comprise the system as well as in the concentrations of the proteins and small molecules external to the system are routinely experienced. In addition to fluctuations in concentration, the enzymatic activities of the proteins of a system may experience fluctuations because of genetic mutations, regulatory covalent modifications, or alternative cellular localizations. Robustness is becoming recognized as an important property of biological systems and in particular biological signal transduction systems; robustness is defined as the property that allows a system to maintain its functions in the face of external and internal perturbations.[1,2] The property of robustness always pertains to specific parameters of the system, and system function may be highly robust to changes in certain parameters while remaining fragile to changes in other parameters. For a signaling system, physiologically important parameters that affect the process of producing an output signal in response to stimulation are the concentrations of proteins and small molecules that comprise the system, and the activities and regulatory properties of the proteins. Some cellular signaling systems are experimentally demonstrated to display robustness to some or all of these parameters,[3,4] and theoretical work argues that such robustness may be a general property of cellular signaling systems and, in particular, the many systems that must function over a broad range of conditions.[5] However, some systems may have specifically evolved to limit robustness to variation of a parameter, to allow that parameter to control the shifting of the system between regulatory regimes. The opposite of a robust system is a fine-tuned one, where the output of the system in response to stimulation depends upon the values of the parameters of the system. Of course, "robustness" and "fine-tuning" are human concepts, and in nature, we expect systems

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to have intermediate properties. For example, system function may tolerate variations of parameters within some limits but be unable to tolerate extreme values such as null.

A common motif of signal transduction systems is the covalent modification cycle, in which the substrate protein of the cycle is subjected to reversible covalent modification that controls its activities. The enzymes that catalyze the modification and demodification of this substrate protein, termed the converter enzymes of the cycle, produce an output signal (the level of the modified substrate protein) in response to a stimulation that regulates one or both converter enzymes. Given the ubiquitous occurrence of covalent modification cycles in nature and their importance in central physiological processes, considerable effort has been focused on theoretical and experimental studies of the signal processing properties of such systems, including studies of signal amplification,[6] noise filtering,[7] and factors affecting sensitivity.[8−10] In this paper, we will demonstrate how a kinetic parameter of the converter enzymes, specifically the effectiveness of the inhibition of one of the converter enzymes, eliminated the robustness of a reconstituted covalent modification cycle toward the concentration of its substrate protein. Furthermore, we were able to document the regulatory catastrophe as the cycle substrate protein concentration was increased beyond the range over which effective signaling occurred, provisionally providing a diagnostic phenotype for the loss of robustness.

The PII-UTase/UR covalent modification cycle is part of two bicyclic cascade systems in *Escherichia coli* that participate in the regulation of nitrogen assimilation (reviewed in ref 11). The PII-UTase/UR-ATase-GS cascade controls the activity of glutamine synthetase (GS) by reversible covalent adenylylation, while the PII-UTase/UR-NRII-NRI cascade controls the phosphorylation state of enhancer-binding transcription factor NRI (NtrC) and, by so doing, regulates the initiation of transcription of nitrogen-regulated genes. In both cascades, the role of the PII-UTase/UR cycle is to communicate the intracellular concentration of glutamine, sensed by UTase/UR, via changes in the uridylylation state of PII. Prior studies of the PII-UTase/UR cycle have revealed the kinetic mechanisms of the UTase and UR activities, elucidated specificity and inhibition constants, established the kinetic mechanism for inhibition by glutamine, and localized the UTase, UR, and glutamine binding activities to specific domains of the protein.[12,13] Our initial observation of the loss of robustness due to alteration of a kinetic parameter of a converter enzyme was quite fortuitous. We examined a His-tagged version of the bifunctional UTase/UR enzyme and were surprised to observe that it had a behavior dramatically different than that of the untagged protein: the His-tagged converter enzyme functioned effectively only in a reconstituted covalent modification cycle when the PII substrate protein concentration of the cycle was very low but not when the PII concentration was high. Comparison of His-tagged and wild-type UTase/UR then revealed which kinetic parameter was responsible for robustness of the system to changes in the concentration of its substrate protein. Further studies using His-tagged enzymes with additional alterations then allowed us to present and test a hypothesis for how the activities of the wild-type enzyme were regulated by the stimulatory effector and how this regulation was defective in the His-tagged enzyme.

MATERIALS AND METHODS

Purified Proteins. The preparations of PII, wild-type UTase/UR, and His-tagged UTase/UR prepared from strain UQ5516 described previously were used.[13−15] A second His-tagged but otherwise wild-type UTase/UR enzyme preparation was obtained (from strain SA1) by metal chelate chromatography as described previously,[13] followed by fractionation on a 300 mL Biogel A1.5 M gel filtration column equilibrated in 50 mM Tris-HCl (pH 7.5), 0.1% EDTA, and 10% (v/v) glycerol. The purified enzyme was dialyzed into storage buffer that was the same as the chromatography buffer, except with 50% (v/v) glycerol. For the sake of clarity, we will distinguish the two different preparations of His-tagged UTase/UR by referring to the strain from which the enzyme was prepared (UQ5516 or SA1). In Figure S1 of the Supporting Information, the appearance after sodium dodecyl sulfate−polyacrylamide gel electrophoresis of wild-type UTase/UR and each of the His-tagged but otherwise wild-type enzymes is shown. Each of these enzymes is approximately 90% pure as judged by visual inspection of the gels. Importantly, the purified enzymes do not appear to be contaminated with ATPases or other activities that interfere with the UT or UR assays (see below). The His-tagged enzymes with alterations in the HD domain [HD-AA (H514A and D515A, from strain UQ5628), HD-QN (H514Q and D515N, from strain UQ5629), and D-HD (Δ-A510−D531, from strain UQ5627)] were also described previously.[13]

Construction and Purification of the D107N Altered Form of UTase/UR. EcoRI and NdeI restriction sites were introduced upstream and a *Bam*HI site was introduced downstream of the *glnD* gene by polymerase chain reaction of plasmid pDOP,[15] using upstream primer CCCGAATTCATATGAATACCCTTCCAGAACAGTAC and downstream primer GGAATTCGGATCCCTGACGTACCGCCGCTGGTGGCCA. The amplified *glnD* gene was cloned into pSelect (Promega), forming pSelect-*glnD*, and this plasmid was mutagenized with the GACGTCAATTTACTGATTTTAAGCCG oligonucleotide. After mutagenesis, the ClaI/NsiI fragment of the mutagenized gene was swapped for the corresponding wild-type fragment in plasmid pglnD9,[15] and then the whole of the mutated *glnD* gene was cloned as an NdeI/EcoRI fragment into NdeI/EcoRI-cleaved pJLA503,[16] resulting in pDOP-D107N. The altered UTase/UR-D107N protein was purified using the same methods that were used for wild-type UTase/UR.[15]

Reconstituted UTase/UR-PII Monocycle. The steady-state levels of PII uridylylation at various glutamine concentrations were measured as described previously.[14] Briefly, reaction conditions included 100 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, 100 mM KCl, 0.3 mg/mL bovine serum albumin, 1 mM DTT, 0.5 mM ATP, 0.2 mM α-ketoglutarate, 0.5 mM [α-^{32}P]UTP, and PII and UTase/UR as indicated. Components except ATP and UTP were combined and prewarmed at 30 °C for 2 min, and reactions were started by addition of a prewarmed mixture containing ATP and UTP. Samples were removed at various times and spotted onto Whatman P81 phosphocellulose filters, which were washed in 5% TCA, dried, and counted by liquid scintillation spectroscopy. Where indicated, AMP-PNP was used in place of ATP. For the determination of steady-state values, long time courses (generally 90 min) were used, and steady states were estimated by averaging values at the latter time samples (generally four samples removed between the 30 and 90 min points of

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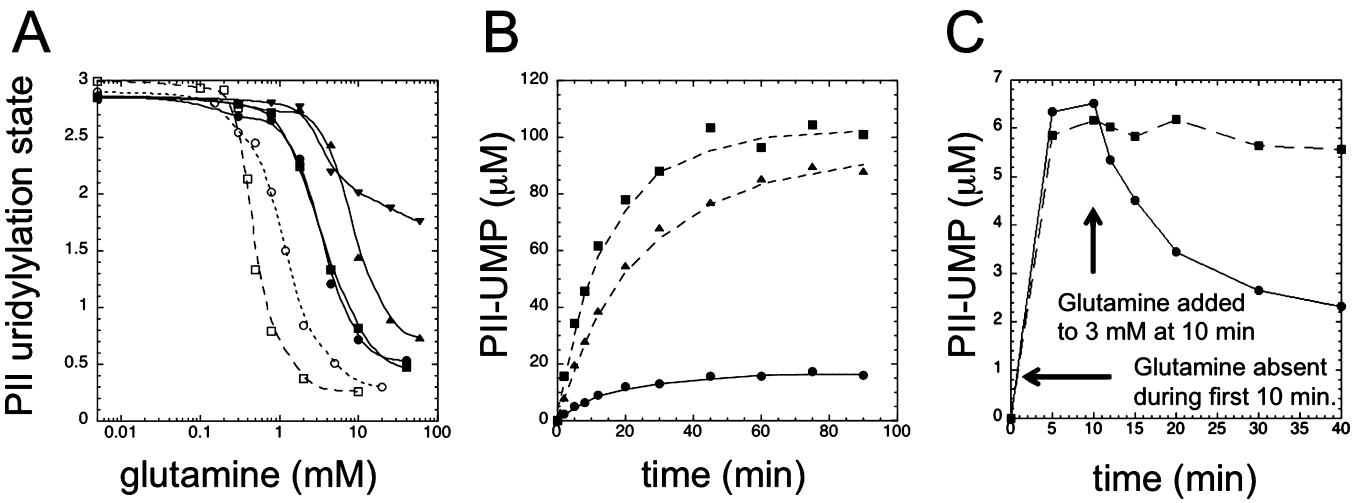

Figure 1. His-tagged version of UTase/UR that was defective in steady-state glutamine signaling in reconstituted PII-UTase/UR covalent modification cycles. (A) Steady-state glutamine responses of reconstituted cycles. Experiments were conducted as described in Materials and Methods, and the steady-state levels of PII modification at various glutamine concentrations are shown, stated in terms of the number of modified subunits per tetramer: (□) 36 μM PII and 1.2 μM wt UTase/UR, (○) 0.5 μM PII and 0.02 μM wt UTase/UR, (▼) 36 μM PII and 1.2 μM His-tagged UTase/UR (UQ5516), (▲) 3 μM PII and 0.1 μM His-tagged UTase/UR (UQ5516), (■) 0.5 μM PII and 0.017 μM His-tagged UTase/UR (UQ5516), and (●) 0.2 μM PII and 0.0067 μM His-tagged UTase/UR (UQ5516). Values plotted on the *y* axis were obtained in the absence of glutamine. (B) Approach to the steady state in reconstituted systems containing 10 mM glutamine. All systems contained 36 μM PII and 1.2 μM enzyme: (●) wt UTase/UR, (▲) His-tagged UTase/UR (UQ5516), and (■) His-tagged UTase/UR (SA1). (C) Response of reconstituted covalent modification cycles to the addition of glutamine. Systems contained 3 μM PII and 0.2 μM enzyme. For the first 10 min, systems were incubated in the absence of glutamine, after which glutamine was added to a final concentration of 3 mM: (●) wt UTase/UR and (■) His-tagged UTase/UR (SA1).

incubation), when the level of PII uridylylation had achieved a constant value. The steady states observed in this work were quite stable. This indicated that the purified proteins were not contaminated by cellular ATPase activity, as depletion of ATP from the reaction mixtures would result in changing levels of PII uridylylation.[22]

Measurement of UT Activity. The initial rate of PII uridylylation was measured as described previously,[14] and the conditions included 100 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, 100 mM KCl, 0.3 mg/mL bovine serum albumin, 0.5 mM ATP or AMP-PNP, as indicated, UMP as indicated, and 0.5 mM [α-^{32}P]UTP. Reaction mixtures lacking ATP (or AMP-PNP) and UTP were incubated for 2 min at 30 °C, and the uridylylation reactions were started by addition of a prewarmed mixture of ATP (or AMP-PNP) and UTP. Samples were removed at various times and spotted onto Whatman P81 phosphocellulose filters, which were washed in 5% TCA, dried, and counted by liquid scintillation spectroscopy. The UT catalytic rate was observed to be almost independent of the enzyme concentration, when it was varied from 0.01 to 1.0 μM, as long as the level of PII was saturating (Figure S2 of the Supporting Information); all of the experiments in this paper were performed within this enzyme concentration range. The assay is accurate because the product can be made highly radioactive and is easily meaured when only a tiny amount of the substrate has been converted, allowing good estimation of initial rates.

Measurement of UR Activity. PII-[^{32}P]UMP was prepared as described previously;[14] briefly, this involved extended incubation of PII with UTase/UR in the absence of glutamine followed by brief heating at 60 °C to inactivate UTase/UR. The initial rate of deuridylylation of PII-UMP was examined at 30 °C as described previously,[14] in reaction mixtures that contained 100 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, 100 mM KCl, 0.3 mg/mL bovine serum albumin, 1 mM (or as indicated) α-ketoglutarate, 0.5 mM ATP or AMP-PNP, as indicated, and glutamine and PII-UMP as indicated. Reaction mixtures were incubated in the absence of PII-UMP for 2 min, and reactions were initiated by addition of prewarmed PII-UMP. Samples were removed at various times and spotted onto Whatman P81 phosphocellulose filters, which were washed in 5% TCA, dried, and counted by liquid scintillation spectroscopy.

The catalytic rates measured in the standard UR assay were observed to depend upon enzyme concentration; the higher the enzyme concentration, the lower the apparent UR k_{cat} in the assay (Figure S2 of the Supporting Information). Furthermore, the assay was variable from day to day, as it was very difficult to provide the substrate PII-UMP at an identical concentration and in an identical modification state. Because of this, meaningful comparisons could be made only in experiments where UR rates were measured side by side at the same enzyme concentration, as we did in this study. This deficiency in the assay is likely due to underestimation of the initial reaction rates; the assay is based upon watching the labeled substrate become unlabeled, and consequently, a significant fraction of the substrate is converted in the assay.

To attempt to obtain more accurate initial rates in the UR assay, we used a thin-layer chromatography method to separate PII-UMP from UMP. This procedure allowed us to measure the product of the UR reaction (UMP) as opposed to simply measuring the disappearance of the PII-UMP substrate in the standard UR assay method. For the TLC-based assay method, reaction conditions were as in the standard UR assay, and 4 μL samples were removed at various times and immediately mixed with 1 μL of 0.5 mM EDTA to stop the reaction. After all samples had been collected, 1 μL aliquots were spotted onto Cellulose PEI thin-layer chromatography plates (J. T. Baker,

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Inc.), and plates were developed using 0.2 M KP_i (pH 8.0) as the solvent. [Prior to the samples being spotted, the positions where samples would be spotted were marked lightly with a pencil, and each plate was chromatographed in water, dried briefly in air, and spotted with 1 μL of a mixture of UTP, UDP, and UMP (30 mM each) that served as a carrier and to indicate the position of UMP.] After development, plates were dried in air and visualized under a hand-held UV light, and the positions of the UMP spots were marked with a pencil. A typical chromatogram that had been marked with a pencil to indicate the nucleotide spots and then subjected to autoradiography is shown in Figure S3 of the Supporting Information. The origin (containing PII-UMP) and the UMP spots were cut from the plates, and the slices were counted by liquid scintillation counting. The fraction of counts appearing in the UMP spot relative to the origin spot was used to calculate the concentration of UMP. This assay method had the advantage that the product of the UR reaction was measured directly, and therefore, it was possible to determine initial rates under conditions where only a small fraction of the initial substrate had been converted. Higher initial rates of catalysis were observed using this method versus the standard assay procedure, which we attribute to the improved estimation of the initial reaction velocities (Figure S2 of the Supporting Information). This TLC-based assay method also showed a dependence of UR catalytic rate on enzyme concentration; while similar k_{cat} values were obtained at 0.01 and 0.1 μM enzyme, much lower than expected k_{cat} values were obtained when the enzyme was 1 μM (Figure S2C of the Supporting Information). Consequently, this UR assay method was also suitable for only side-by-side comparisons of different enzyme samples at identical enzyme concentrations.

RESULTS

The UTase/UR-PII Covalent Modification Cycle Is Robust to Changes in the PII Concentration. Using purified proteins, we studied the responses of the UTase/UR-PII cycle to glutamine in systems that contained different concentrations of PII and UTase/UR. We show elsewhere that good glutamine signaling properties were obtained when 100 μM PII was used, the highest concentration we were able to provide, and that fairly low enzyme concentrations did not prevent effective signaling, although when the enzyme concentration was low relative to that of PII, the reaction mixtures had to be incubated for a very long time to obtain the steady-state level of PII uridylylation.[17] We will also show elsewhere that variation of PII and enzyme concentrations alters the sensitivity of responses and the midpoint of responses;[17] such effects are well-known in theory.[8−10,18] For the purposes of this study, we focus on a fairly narrow range of PII concentrations at which the wild-type system displayed excellent responsiveness to glutamine (Figure 1A). PII is a homotrimeric protein that can be reversibly modified at a unique site (Y51) on each subunit, such that its modification state can range from zero to three modifications per trimer. When the PII concentration was 36 μM and the UTase/UR concentration was 1.2 μM, the PII modification state went from 2.99 uridylyl groups in the absence of glutamine to 0.26 uridylyl group per trimer in the presence of 10 mM glutamine (Figure 1A); therefore, the range of modification states sampled during this transition was ∼2.73 of a possible range of 3. This range was highly reproducible; in four additional repeats of the experiment, the range of PII modification states varied from 2.69 to 2.73. Because PII is a homotrimer and UTase/UR is a monomer, at this ratio of substrate to enzyme there were 90 PII modification sites for every converter enzyme active site. The range of PII modification states was only slightly diminished when the PII concentration was 0.5 μM (low value) and the UTase/UR concentration was 0.02 μM, in which case the range of uridylylation states was ∼2.59 (Figure 1A). At this substrate to enzyme ratio, there were 75 substrate sites per converter enzyme active site. In four repeats of the experiment under these conditions, and in one additional experiment where the PII concentration was at 0.5 μM and the UTase/UR concentration was 0.05 μM, the range of uridylylation states was again found to be quite consistent, varying between 2.53 and 2.61.

The two conditions noted so far were chosen to ensure that both ultrasensitive and hyperbolic regimes were sampled (Figure 1A), and the variation of conditions discernibly shifted the midpoint of the response, as expected.[17] These differences notwithstanding, a wide range of steady-state uridylylation states was consistently obtained in response to changes in the glutamine concentration, regardless of whether the PII concentration was high or low.

A His-Tagged Version of UTase/UR Displayed an Altered Response to Glutamine in Reconstituted Monocycles. We examined two different preparations of a His-tagged version of UTase/UR that were produced by cloning the *glnD* structural gene into common expression vector pET15b.[13] The two preparations of the enzyme were made in our two laboratories, using slightly different procedures (Materials and Methods). For our studies, we conducted experiments shown here with both enzyme preparations, which behaved the same and are mentioned by the strain that served as the source of the enzyme: UQ5516 (Wisconsin) and SA1 (Michigan). The altered form of the enzyme resulting from expression from pET15b contains the sequence Met-Gly-Ser-Ser-His-His-His-His-His-His-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His added to the N-terminus of the protein. The His-tagged UTase/UR (UQ5516), when used at 1.2 μM, provided a very shallow response to glutamine when the PII concentration was 36 μM, and the midpoint of the response was shifted to a significantly higher glutamine concentration, relative to the results obtained with the wild-type enzyme under the same conditions (Figure 1A). In this experiment, a narrow range of uridylylation states (∼1.1) signaled glutamine concentration, and this narrow range was biased toward high uridylylation states. Using the His-tagged UTase/UR UQ5516 enzyme preparation, we also examined three other conditions where the ratio of PII modification sites to enzyme active sites was also 90:1, specifically, systems where the enzyme concentration was 0.1 μM and where the PII concentration was 3 μM, where the enzyme concentration was 0.017 μM and the PII concentration was 0.5 μM, and where the enzyme concentration was 0.0067 μM and the PII concentration was 0.2 μM (Figure 1A). In the system where the PII concentration was 3 μM, a wider range of uridylylation states signaled glutamine concentration (∼2.1) than when the PII concentration was 36 μM, and when the PII concentration was 0.5 and 0.2 μM, a still wider range of uridylylation states signaled the glutamine concentration (∼2.4), almost equal to the range obtained when wild-type UTase/UR (lacking the His tag) was used (Figure 1A). These results suggested that the absolute PII concentration was the important parameter controlling the range of uridylylation states obtained, and not the ratio of PII to the enzyme (which

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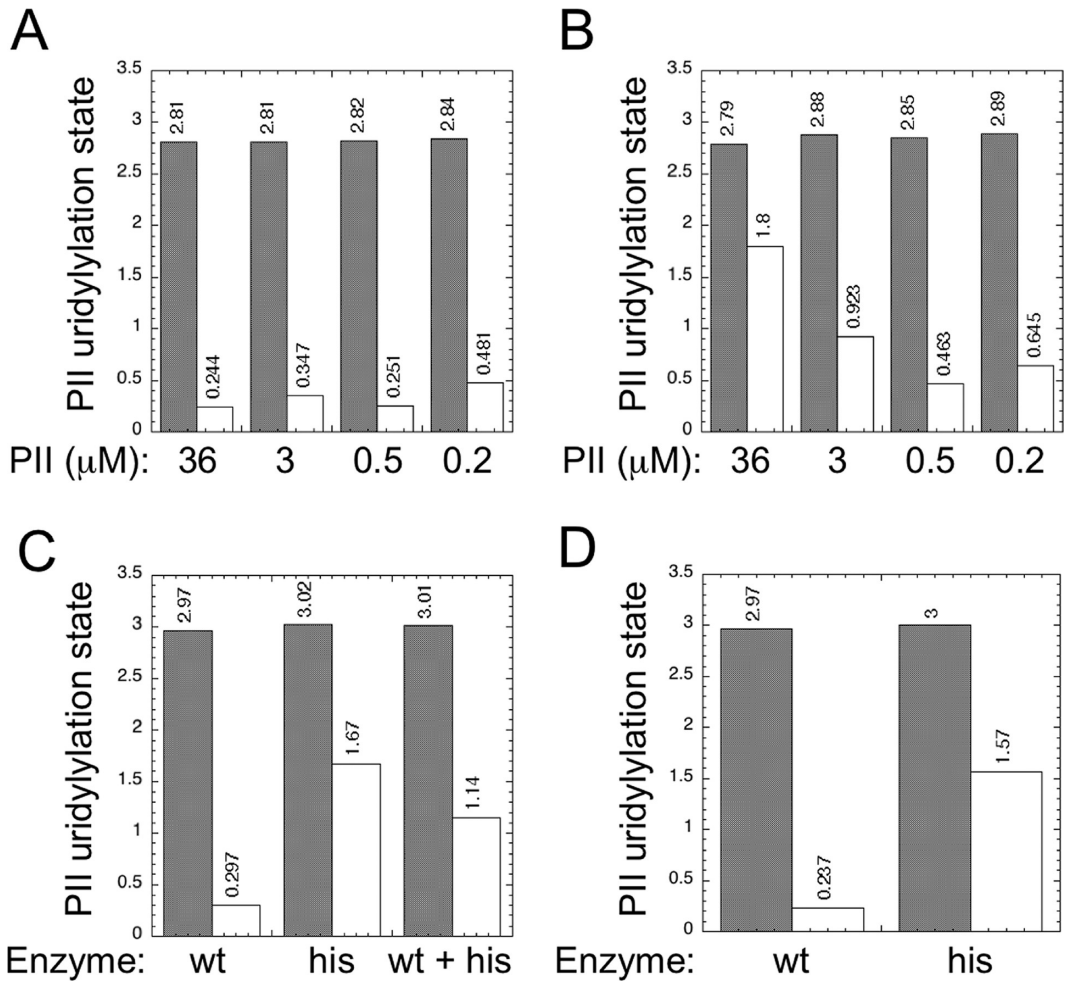

Figure 2. Comparison of steady-state PII modification levels in reconstituted covalent modification cycles containing wild-type or His-tagged UTase/UR. Results of experiments from which glutamine was absent are shown with filled bars; results of experiments in which the glutamine concentration was 10 mM are shown with empty bars. (A) Results for systems containing wild-type UTase/UR. (B) Results for systems containing His-tagged UQ5516 UTase/UR. Experiments for panels A and B for each concentration of PII were performed side by side as described in the text. To maintain a fixed ratio of PII to catalytic sites in the set of experiments, when the PII concentration was 36 μM, the UTase/UR concentration was 1.2 μM; when the PII concentration was 3 μM, the UTase/UR concentration was 0.1 μM; when the PII concentration was 0.5 μM, the UTase/UR concentration was 0.017 μM; and when the PII concentration was 0.2 μM, the UTase/UR concentration was 0.0067 μM. (C) Result of combining wild-type and His-tagged enzymes. The PII concentration was 36 μM, and wt and/or His-tagged enzymes were present at 1.2 μM each, as indicated. (D) Steady-state levels of PII uridylylation in systems where AMP-PNP was present instead of ATP. Experiments were conducted as described for panel A and panel B, where the PII concentration was 36 μM and the UTase/UR concentration was 1.2 μM, except that ATP was replaced by 0.5 μM AMP-PNP.

was fixed at 90:1). In addition to affecting the range of uridylylation states obtained in response to glutamine variation, the PII concentration also controlled the midpoint of the glutamine response (Figure 1A).

The experiments presented in Figure 1A using wild-type and His-tagged UTase/UR were performed on different occasions, and because of the complexity of the experiments, it is highly desirable to have side-by-side comparisons performed under identical conditions. In Figure 1B, we show the approach to the steady state in side-by-side experiments for systems containing 10 mM glutamine, 36 μM PII, and 1.2 μM enzyme (90:1 ratio of PII modification sites to UT and UR active sites). When the wild-type enzyme was used, a low PII uridylylation-state level was obtained, whereas when the His-tagged enzymes were used, a high PII uridylylation-state level was obtained (Figure 1B). His-tagged UTase/UR prepared from strain SA1 resulted in slightly higher PII uridylylation-state levels than when His-tagged UTase/UR prepared from strain UQ5516 was used, and this behavior was consistently obtained, as shown in the experiments to follow and numerous additional experiments. Differences of this magnitude were also obtained in activity measurements of wild-type UTase/UR preparations made on different occasions and probably reflect differences in the loss of activity during purification (Figure S4 of the Supporting Information). The high steady-state levels of PII uridylylation obtained with the His-tagged enzymes in the presence of 10 mM glutamine are consistent with the results from Figure 1A.

In another experiment using reconstituted covalent modification cycles, we explored the effect of allowing the systems to reach the steady state in the absence of glutamine, such that PII was highly uridylylated, and then adding glutamine to 3 mM, a concentration expected to result in an intermediate level of PII uridylylation (Figure 1B). For these experiments, the trimeric PII concentration was 3 μM and the enzyme concentration was 0.2 μM, such that the ratio of substrate to catalytic sites was 45:1. When the system contained wild-type

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UTase/UR, addition of glutamine resulted in an immediate decrease in the level of PII uridylylation and the system approached the steady state characteristic of the final glutamine concentration and conditions (Figure 1C). This experiment was repeated with three independent preparations of wild-type UTase/UR with similar results (Figure S4B of the Supporting Information). By contrast, when the system contained His-tagged UTase/UR (SA1), the addition of glutamine was essentially without effect and the PII uridylylation-state level remained high (Figure 1C). This experiment was repeated on another occasion with a similar result. Together, the experiments in Figure 1 indicated that the His-tagged enzymes displayed a severe defect in glutamine signaling when used in reconstituted covalent modification cycles, particularly when the PII was present at a relatively high concentration of 3 or 36 μM.

Additional side-by-side experiments were used to compare the wild-type and His-tagged enzymes in reconstituted covalent modification cycles at different PII and enzyme concentrations, and in the presence or absence of 10 mM glutamine. In Figure 2, we show a series of experiments in which the wild-type enzyme and the His-tagged (UQ5516) enzyme were examined at a fixed ratio of PII subunits to enzyme active sites of 90:1. Although for the sake of clarity the data are presented in separate panels for the wild-type enzyme (Figure 2A) and the His-tagged (UQ5516) enzyme (Figure 2B), the experiments were performed side by side for both enzymes for each PII concentration; in addition, all experiments with the exception of those using 3 μM PII were also performed side by side with the SA1 His-tagged preparation, and those data are not shown only because they are quite similar to the data for the His-tagged enzyme prepared from UQ5516. As shown in Figure 2A, the wild-type system was robust to changes in the PII concentration when the ratio of substrate to enzyme was held constant. By contrast, the system containing the His-tagged enzyme was not robust to PII concentration over the same range (Figure 2B), even though the ratio of substrate to enzyme was held constant. At high PII concentrations, the systems with the His-tagged enzymes were unable to maintain a low level of PII modification in the presence of 10 mM glutamine (shown for UQ5516 in Figure 2B). Another consistent, but less dramatic, result was that for all three enzyme samples (wild type, UQ5516, and SA1), a slightly higher PII uridylylation-state level was obtained in the presence of glutamine when the PII concentration was 0.2 μM, relative to that obtained when the PII concentration was 0.5 μM [shown for wild-type and His-tagged UTase/UR (UQ5516) in Figure 2].

In another side-by-side comparison, we examined systems that contained 36 μM PII and 1.2 μM wild-type or His-tagged (UQ5516) enzyme, or a combination of both enzymes at 1.2 μM each, with or without 10 mM glutamine (Figure 2C). Again, the wild-type system displayed an excellent response to glutamine, whereas the system containing the His-tagged (UQ5516) enzyme displayed elevated levels of PII modification at 10 mM glutamine. The system containing both enzymes produced a level of PII-UMP at 10 mM glutamine that was intermediate between the levels obtained with either of the two enzymes. This suggested that neither enzyme preparation contained an activator or inhibitor but rather that the enzyme catalytic rates were balanced against one another.

The results described so far could have been explained by a deficiency in the UR activity of the His-tagged enzyme, by a defect in activation of the UR activity by glutamine, or by a defect in the ability of glutamine to inhibit the UT activity of the enzyme. Such defects could result in elevated levels of PII modification states in the presence of glutamine. However, the mechanism for explaining this defect must also account for its dependence on the PII concentration at a fixed ratio of PII to enzyme.

The two activities of the UTase/UR enzyme are of disproportionate strength; the UT activity has a k_{cat} of ~144 min^{-1} in the absence of glutamine, while the UR activity displays a k_{cat} of only ~6 min^{-1} in the presence of 10 mM glutamine, when measured in our standard assay.[12] Furthermore, the UR activity has a basal k_{cat} of ~2 min^{-1} in the absence of glutamine, such that it is only regulated ~3-fold by glutamine.[12] By contrast, the UT activity is strongly inhibited by glutamine;[12] we show later in this paper that under the conditions of the experiments performed here we realized ~100-fold inhibition. In the course of our work, we observed that the UR activity of UTase/UR was increased when ATP in the reaction mixtures was replaced by AMP-PNP (Figure S5 of the Supporting Information) (the adenylylate nucleotide in the reaction mixtures is a ligand of PII[19] and is required for the interaction of PII with UTase/UR). Also, inhibition of the UT activity by glutamine was normal when AMP-PNP replaced ATP in the reaction mixtures (Figure S6 of the Supporting Information). Therefore, by replacing ATP with AMP-PNP, we could modestly elevate the UR activity while retaining good regulation of the UT activity of the systems. We examined reconstituted UTase/UR-PII monocycles containing AMP-PNP in place of ATP, again using a PII concentration of 36 μM and an enzyme concentration of 1.2 μM so that the substrate:enzyme ratio was again 90:1 (Figure 2D). Under these conditions, we obtained results that were quite similar to those obtained when the systems contained ATP; that is, the system containing the wild-type enzyme displayed good regulation by glutamine, whereas the system containing the His-tagged (UQ5516) enzyme had elevated levels of PII modification at 10 mM glutamine (Figure 2D). The His-tagged enzyme prepared from strain SA1 was also examined side by side in this experiment, and the results were essentially the same as for the enzyme purified from strain UQ5516 (not shown). These results showed that a modest increase in the UR activity had little effect in systems containing either the wild-type or His-tagged enzymes and provided a clue that a major factor in controlling the PII modification state was the inhibition of the powerful UT activity by glutamine.

A Combination of His-Tagged UTase/UR and an Altered Form of the Enzyme Displaying Only UR Activity Effectively Regulated the PII Uridylylation State in Response to Glutamine. Our conclusion from the experiment shown in Figure 2D was that a modest increase in UR activity (caused by inclusion of AMP-PNP in the reaction mixtures) was not sufficient to allow changes in the glutamine concentration to bring about broad changes in the steady-state levels of PII modification when the enzyme bore a His tag. We next examined whether a larger increase in the level of UR activity in the reaction mixtures might allow effective glutamine signaling. To test this, we used an altered form of UTase/UR containing the D107N mutation, constructed and purified as described in Materials and Methods. This altered enzyme is similar to previously described altered versions of UTase/UR containing different D107 substitutions,[13] except that it does not contain a His tag. The D107 residue is one of the two critical Mg^{2+}-chelating aspartate residues of the

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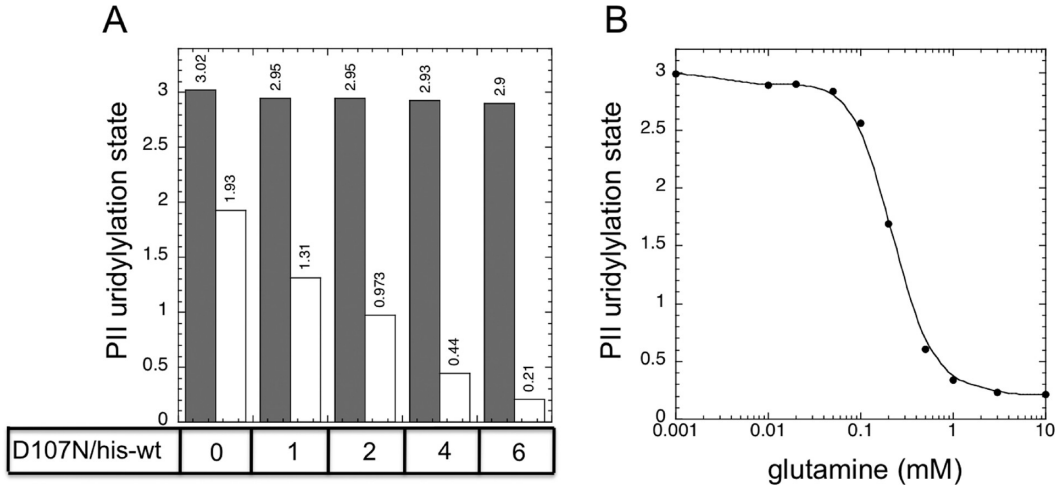
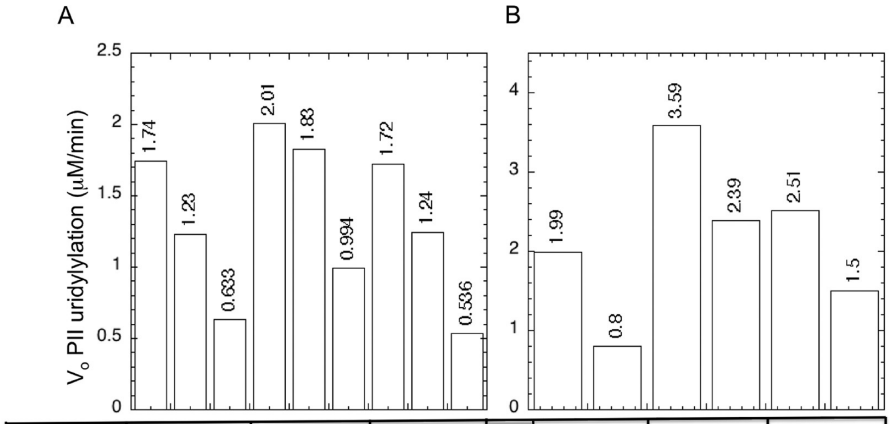


Figure 3. Combination of His-tagged UTase/UR and an altered form of the enzyme displaying only UR activity effectively regulated PII uridylylation state in response to glutamine. (A) Steady-state PII uridylylation state in the absence of glutamine (filled bars) and in the presence of 10 mM glutamine (empty bars). The PII concentration was 36 μM; the His-tagged UTase/UR (UQ5516) concentration was 0.5 μM, and the altered D107N UTase/UR concentration was 0, 0.5, 1, 2, or 3 μM to provide the indicated ratios of enzymes. (B) Glutamine signaling by a reconstituted UTase/UR-PII cycle containing a combination of His-tagged UTase/UR (UQ5516) (0.5 μM) and the altered D107N UTase/UR (3 μM). The PII concentration was 36 μM. The value plotted on the *y* axis was obtained in the absence of glutamine.



Enzyme	wild type			UQ5516			SA1			wild type	UQ5516	SA1
Enz (μM)	.02	.02	1.0	.02	.02	.2	.02	.02	.2	.05	.05	.05
PII (μM)	5	36	36	5	36	36	5	36	36	36	36	36
Gln (mM)	0	0	10	0	0	10	0	0	10	0 .1	0 .1	0 .1

Figure 4. Initial rate of PII uridylylation (UT activity) and its regulation by glutamine and UMP. Measurement of the initial rate of PII uridylylation was as described in Materials and Methods.

conserved NT domain, and the purified D107N enzyme did not display measurable UT activity. We will characterize the UR activity of this protein in later sections of this report; as we will show, this activity and its regulation by glutamine were nearly the same as for the wild-type enzyme.

We examined the uridylylation state of PII, present at the high concentration of 36 μM, in the presence and absence of 10 mM glutamine, when His-tagged UTase/UR (UQ5516) was present at a concentration of 0.5 μM and the D107N altered enzyme was present at various concentrations (Figure 3A). When the His-tagged (UQ5516) enzyme was the only enzyme present, the PII uridylylation state only varied over a narrow range, consistent with the results shown in Figures 1A and 2B, but the combination of the His-tagged UTase/UR and the altered D107N enzyme resulted in more effective glutamine signaling (Figure 3A). When the D107N enzyme was present at a concentration of 3 μM in combination with the His-tagged UTase/UR at a concentration of 0.5 μM (such that the D107N:His-tagged enzyme ratio was 6:1), the PII uridylylation state varied from 2.9 in the absence of glutamine to 0.21 in the presence of 10 mM glutamine, for a range of ~2.7, as typically seen with wild-type (untagged) UTase/UR (Figure 1A). When the steady-state responses to a wide range of glutamine concentrations were examined under these conditions, very effective glutamine signaling was observed (Figure 3B), reminiscent of the glutamine signaling by the wild-type (untagged) enzyme under similar conditions (Figure 1A). Thus, in practice, the signaling defect of the His-tagged UTase/UR enzyme could be offset simply by addition of the

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monofunctional D107N enzyme that provided additional UR activity (Figure 3B).

UT Activity of the His-Tagged Enzymes. A series of preliminary experiments indicated that both preparations of His-tagged but otherwise wild-type UTase/UR had normal levels of UT activity, which was regulated by glutamine, and displayed K_m values for PII and substrate inhibition by PII similar to those of the wild-type enzyme (Figure S7A of the Supporting Information).[15] The His-tagged enzyme preparations displayed a slightly higher inhibition constant for glutamine (0.14 mM) than did the wild-type enzyme (0.06 mM), as shown for the wild-type and UQ5516 enzyme preparations in Figure S7B of the Supporting Information. Notably, inhibition of the UT activity His-tagged enzyme was incomplete at 10 mM Gln, whereas the wild-type enzyme was almost completely inhibited at this glutamine concentration (Figure S7B of the Supporting Information).

In Figure 4, we show side-by-side comparisons of the wild-type and His-tagged enzymes under various conditions to highlight the similarities and differences of these enzymes. For all three enzyme samples, we measured the initial rate of PII uridylylation (UT activity) in the presence and absence of 10 mM glutamine with 36 μM PII. Because the UT activity is inhibited by glutamine, the experiments conducted in the presence of glutamine utilized elevated levels of enzyme to obtain easily measurable catalytic rates, as shown in Figure 4. This procedure was used because we verified that the k_{cat} of the UT reaction was largely independent of the enzyme concentration, as long as the PII substrate concentration was saturating (Figure S2 of the Supporting Information). We also measured the UT activity when the PII concentration was 5 μM and glutamine was absent to allow comparison with the rates obtained at 36 μM PII and an assessment of PII substrate inhibition (Figure 4).

When the PII concentration was 36 μM, the UT activity of wild-type UTase/UR was regulated ~97.2-fold by glutamine (Figure 4). The enzyme displayed substrate inhibition by PII in the absence of glutamine, as the catalytic rate was faster with 5 μM PII than it was with 36 μM PII. The estimate of 97.2-fold regulation from Figure 4 was determined as follows: when the PII concentration was 36 μM, an enzyme concentration of 0.02 μM provided a rate of 1.23 μM min^{-1} in the absence of glutamine (corresponding to a k_{cat} of 61.5 min^{-1}) and an enzyme concentration of 1 μM provided a rate of 0.663 μM/min in the presence of 10 mM glutamine (corresponding to a k_{cat} of 0.633 min^{-1}). Thus, glutamine lowered the k_{cat} 97.2-fold.

In contrast to the wild-type enzyme, the His-tagged UTase/UR preparations (SA1 and UQ5516) were regulated only 18- and 23-fold, respectively, by 10 mM glutamine (Figure 4A). Thus, when the PII concentration was 36 μM, 0.2 μM enzyme provided approximately half the activity when glutamine was present than did the enzyme at 0.02 μM in the absence of glutamine (Figure 4A). These results show that the His-tagged enzyme preparations had a significant defect in glutamine inhibition of the UT activity. In the absence of glutamine, the UT activities of the His-tagged enzymes were similar to that of the wild type, but in the presence of glutamine, the UT activity of the His-tagged enzymes was dramatically higher (Figure 4A).

To allow the accurate assessment of the glutamine regulation of these enzyme samples at fixed enzyme levels, we examined the effect of 0.1 mM glutamine on the initial rate of uridylylation when the PII concentration was 36 μM and the enzyme concentration was 0.05 μM (Figure 4B). As shown, glutamine regulation of the wild-type enzyme was sharper than that obtained with the His-tagged enzyme preparation; the level of inhibition of the wild-type activity was 59.8%, while the UQ5516 preparation was inhibited 40.2% and the SA1 enzyme preparation 33.6% (Figure 4B). Together, the results in Figure 4 show that the His-tagged enzymes had a significant defect in glutamine regulation of the UT activity when the PII concentration was 36 μM.

In another set of side-by-side experiments, the glutamine regulation of the UT activity of the wild-type and His-tagged (UQ5516) enzymes was examined in experiments where the PII concentration was 5 μM and the enzyme concentration was 0.05 μM (Figure 5). Under these conditions, the difference in

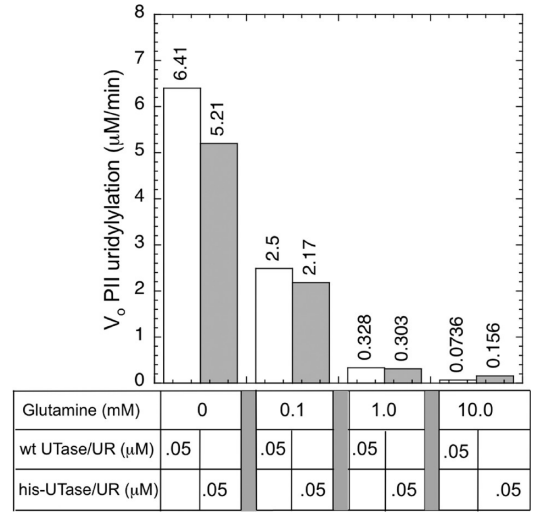

Figure 5. Initial rate of PII uridylylation and its regulation by glutamine and UMP. The initial rate of PII uridylylation was measured as described in Materials and Methods, with 5 μM PII. The His-tagged enzyme used in this experiment was the UQ5516 preparation.

glutamine regulation of the enzymes was discernible but was less dramatic; the wild-type enzyme was regulated 87-fold by 10 mM glutamine, while the His-tagged UQ5516 enzyme preparation was regulated 33-fold by 10 mM glutamine (Figure 5). Similar subtle differences were observed in the regulation by lower concentrations of glutamine under these conditions (Figure 5). For example, when the glutamine concentration was 0.1 mM, the wild-type enzyme was inhibited 61% while the His-tagged UQ5516 enzyme was inhibited 58.3% (Figure 5). This side-by-side comparison was repeated on another occasion; the wild-type enzyme was inhibited 59.9% by 0.1 mM glutamine, while the His-tagged UQ5516 enzyme was inhibited 57.4% by 0.1 mM glutamine. We conclude that when the PII concentration was 5 μM, glutamine regulation of the wild-type and His-tagged enzymes was nearly the same. By comparison, there was an obvious distinction in the glutamine regulation of the wild-type and His-tagged enzymes when the PII concentration was 36 μM (Figure 4).

UR Activity of Enzymes. A series of preliminary experiments using the standard UR assay (Materials and Methods) indicated that the His-tagged enzymes displayed a modest defect in catalyzing the deuridylylation of PII-UMP, relative to that of the wild-type enzyme. In Figure 6A, we present side-by-side comparisons of the enzymes, in which the initial rate of PII-UMP deuridylylation was measured in reaction mixtures that contained ATP as the adenylylate

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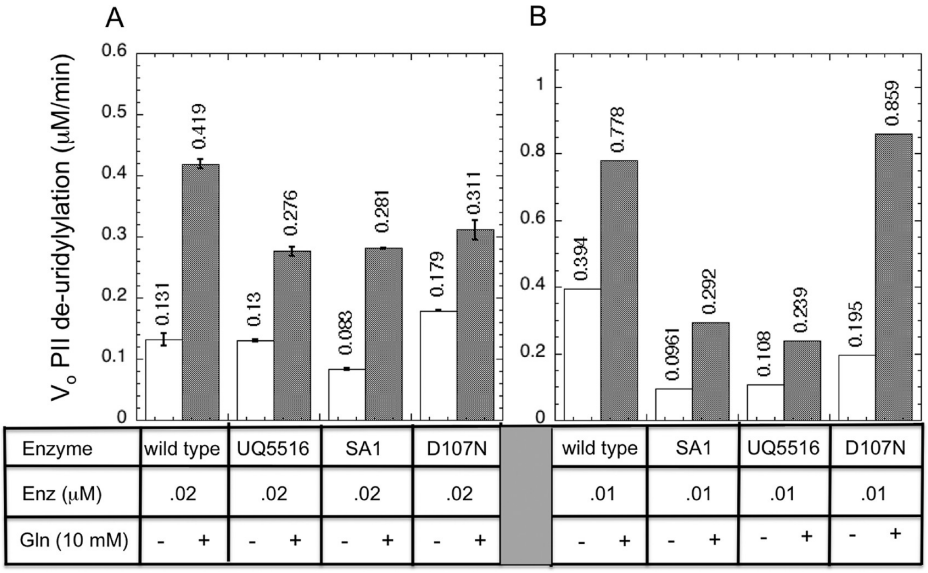

Figure 6. UR activity of wild-type UTase/UR, His-tagged UTase/UR, and altered D107-N UTase/UR, and its regulation by glutamine in systems containing ATP. (A) Measurements using the standard assay protocol. Initial rates of PII-UMP deuridylylation were determined as described in Materials and Methods, in experiments where uridylylated PII subunits were initially present at a concentration of 4 μM. Error bars indicate the standard deviation for duplicate trials, which were performed the next day. (B) Measurements using the TLC-based assay protocol. Initial rates of PII-UMP deuridylylation were determined as described in Materials and Methods, in experiments where uridylylated PII subunits were initially present at a concentration of 10.33 μM.

nucleotide and contained 4 μM PII-UMP and 0.2 μM enzyme. Under these conditions, the wild-type enzyme displayed a basal UR activity, which was stimulated 3.2-fold by 10 mM glutamine, as expected.[19] The UR activity is weak, and the stimulated rate for the wild-type enzyme in this experiment corresponded to ~2.1 min^{-1}. By comparison, the His-tagged enzymes displayed a similar basal UR rate, and 2−3-fold stimulation by 10 mM glutamine (Figure 6). The D107N enzyme displayed a slightly higher basal UR activity than wild-type UTase/UR, and this rate was stimulated <2-fold by glutamine; as a result, the stimulated UR rate was slightly lower than that obtained with the wild-type enzyme (Figure 6A). On the basis of the data in Figure 6A, it appeared that the His-tagged enzymes had an only modest defect in the UR activity.

Because of concerns about the accuracy of the standard UR assay, we developed an alternative assay procedure incorporating a thin-layer chromatographic separation of the reaction product (UMP), as described in Materials and Methods. Although this new assay procedure is labor-intensive, we believe it allows more accurate estimations of the initial reaction rates, particularly when higher levels of activity are being measured. As shown in Figure 6B, the His-tagged enzymes displayed a significant (~3-fold) defect in the level of UR activity, while the D107N enzyme was again quite similar to the wild-type enzyme. Also, higher UR activities were obtained using the TLC-based assay method as compared to the standard assay method (panel A vs panel B of Figure 6).

As noted above, the UR activity is stimulated when the ATP in the reaction mixtures is replaced with AMP-PNP. We compared the UR activity of the wild-type and His-tagged (SA1) enzymes in the presence of AMP-PNP in side-by-side experiments that were repeated; the His-tagged enzyme displayed a 5-fold lower level of basal UR activity and a 2.2-fold lower level of glutamine-activated UR activity in comparison to the wild-type enzyme (Figure S8 of the Supporting Information).

A Hypothesis To Explain PII Substrate Inhibition of UT Activity, Glutamine Regulation of UT and UR Activities, and the Effect of Adding a His Tag to UTase/UR on the Robustness to PII Concentration. UTase/UR is a monomeric protein, consisting of four functional domains (depicted schematically in Figure 7).[13] By analogy with other

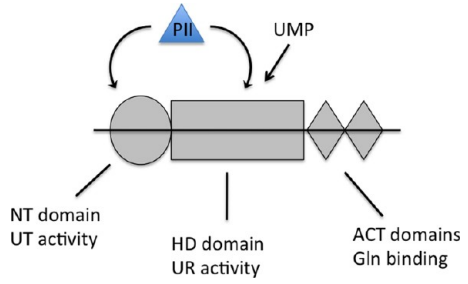

Figure 7. Schematic depiction of the domain arrangement of UTase/UR and the sites from which UMP and PII exert their inhibitory effects. The N-terminal NT domain of UTase/UR is depicted as a gray circle and the central HD domain of UTase/UR as a gray rectangle, and the tandem C-terminal ACT domains of UTase/UR are depicted as gray diamonds. PII, depicted as a blue triangle, binds to the NT domain at the site where it is uridylylated (substrate site) and binds to the HD domain at the site where it is formed from PII-UMP (product site). UMP is also a product of the UR activity of the HD domain.

proteins containing ACT domains, it is likely that binding of glutamine to the tandem ACT domains at the C-terminal end of the protein is responsible for inhibition of the UT activity and stimulation of the UR activity. Alterations of the ACT domains block glutamine regulation of the UT and UR activities.[13] Because the UT activity is tightly regulated by glutamine, we hypothesize that the HD domain is not only responsible for catalysis of the UR activity but also responsible for controlling the UT activity in response to glutamine binding. That is, the HD domain has a signal transduction

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function. The only other possible mechanisms for regulation of the UT activity by binding of glutamine to the ACT domains would be for the ACT domains to contact the N-terminal UT domain directly, such as if the protein had an overall curvature or formed an oligomer in which the ACT domains of one subunit were in contact with the NT domain of the opposing subunit. Both of these possibilities seem less likely than if the signal of glutamine binding is passed to the NT (UT) domain by the UR domain. Earlier studies with the related ATase enzyme that reversibly modifies GS showed that in that protein the two catalytic domains regulated each other and had both catalytic and signal transducing functions,[19,20] as we hypothesize for UTase/UR.

Because PII is a product of the UR reaction catalyzed by the central HD domain and is the substrate of the UT activity of the NT domain, there are two binding sites on UTase/UR for PII (Figure 7). We hypothesize that PII exerts substrate inhibition of the UT activity upon binding to its site in the HD domain. We also hypothesize that the unusual properties of His-tagged UTase/UR resulted from an abnormal interaction between the N-terminal nucleotidyltransferase (NT) domain and the central HD domain. Because of this abnormal domain arrangement, the binding of PII to the HD domain (the substrate inhibition site) interferes with the transmission of the glutamine signal from the ACT domains to the NT domain and weakens the inhibition of the UT activity by glutamine.

A Small Deletion within the HD Domain Eliminated Glutamine Regulation of UT Activity and Also Eliminated Substrate Inhibition of UT Activity by PII. If the PII-mediated substrate inhibition of UT activity was due to binding of PII to the UR active site within the HD domain of UTase/UR, then mutations that alter the UR active site are predicted to eliminate substrate inhibition. The Δ-HD protein (purified from strain UQ5627)[12] is a His-tagged protein in which 22 residues have been removed by a deletion within the HD domain (Δ-A510–D531), and this protein lacks UR activity.[13] This protein displayed fairly weak UT activity, and this activity was not regulated by glutamine[13] (Figure 8). Consistent with our hypothesis, the UT activity of the Δ-HD protein did not display substrate inhibition by PII. We interpret

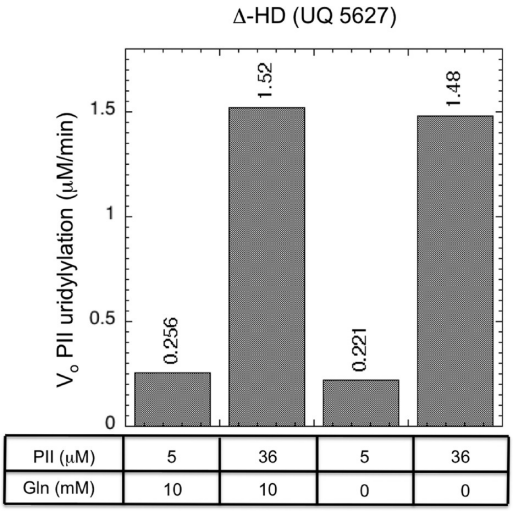

Figure 8. UT activity of the His-tagged Δ-HD (UQ5627) protein. Initial rates of PII uridylylation were determined as described in Materials and Methods.

the absence of glutamine regulation of the UT activity resulting from the small deletion within the HD domain as reflecting a loss of the signal transduction function of the HD domain.

Amino Acid Substitutions of the Catalytic Residues within the HD Domain Eliminated Substrate Inhibition of UT Activity by PII and Partially Restored Glutamine Regulation of UT Activity. The HD-AA (UQ5628) and HD-QN (UQ5629) proteins are His-tagged enzymes that contain amino acid substitutions at the conserved H and D residues of the HD domain.[13] These proteins lack UR activity but display considerable UT activity, which was regulated by glutamine[13] (see below). We observed that these proteins displayed better regulation of UT activity by glutamine than did the His-tagged but otherwise wild-type versions of UTase/UR (Figure 9). When the PII concentration was 36 μM and UMP was absent, each of these proteins displayed 42-fold regulation of UT activity by glutamine (Figure 9). That is, when glutamine was absent and the enzyme concentration was 0.02 μM, the activity was more than 4-fold higher than when glutamine was present and the enzyme concentration was 0.2 μM. When the PII concentration was 5 μM, the HD-AA protein displayed 59-fold regulation by glutamine and the HD-QN protein displayed 85-fold regulation by glutamine. In additional experiments, we also observed that these two His-tagged proteins with HD domain alterations displayed better regulation than did the His-tagged but otherwise wild-type UTase/UR when glutamine was present at a low concentration of 0.1 mM. Thus, remarkably, alterations within the HD domain partially compensated for the presence of the His tag in allowing strong glutamine control of UT activity. Neither the HD-AA protein nor the HD-QN protein displayed substrate inhibition of UT activity by PII (Figure 9), which is consistent with our hypothesis.

A Reconstituted UTase/UR-PII Monocycle Comprised of Monofunctional UTase and UR Enzymes. Because the HD-AA protein lacked UR activity, we used this protein along with the D107N monofunctional UR enzyme to produce a reconstituted UTase/UR-PII cycle comprised of two monofunctional enzymes. The altered HD-AA protein lacked substrate inhibition by PII, and its UT activity was well-regulated by glutamine even when the PII concentration was high; we therefore expected that a monocycle containing the monofunctional HD-AA and D107N enzymes should be robust to PII concentration. To examine this, we held the PII concentration fixed at 36 μM and examined the effect of combining 0.8 μM HD-AA enzyme with various concentrations of the D107N enzyme. The steady-state uridylylation of PII was assessed in systems lacking glutamine or containing 10 mM glutamine, to discern the range of uridylylation states that could be obtained in response to glutamine (Figure 10). As shown, the largest range of uridylylation states in response to glutamine signaling was obtained in this experiment when the ratio of D107N to HD-AA was 5. Below this ratio, a narrow range of uridylylation states was obtained, biased toward higher uridylylation states, while at a higher ratio of enzymes, a narrow range of uridylylation states was obtained that was biased toward lower uridylylation states (Figure 10A). We therefore explored using a modest excess of the D107N enzyme, relative to the HD-AA enzyme in reconstituted monocycles, and in additional experiments found that a 4:1 ratio of the two enzymes gave the optimal responsiveness to glutamine. The results of using a 4:1 D107N:HD-AA ratio in a reconstituted UTase/UR-PII monocycle are shown in Figure 10B. A PII concentration of 36 μM was used, and unlike the

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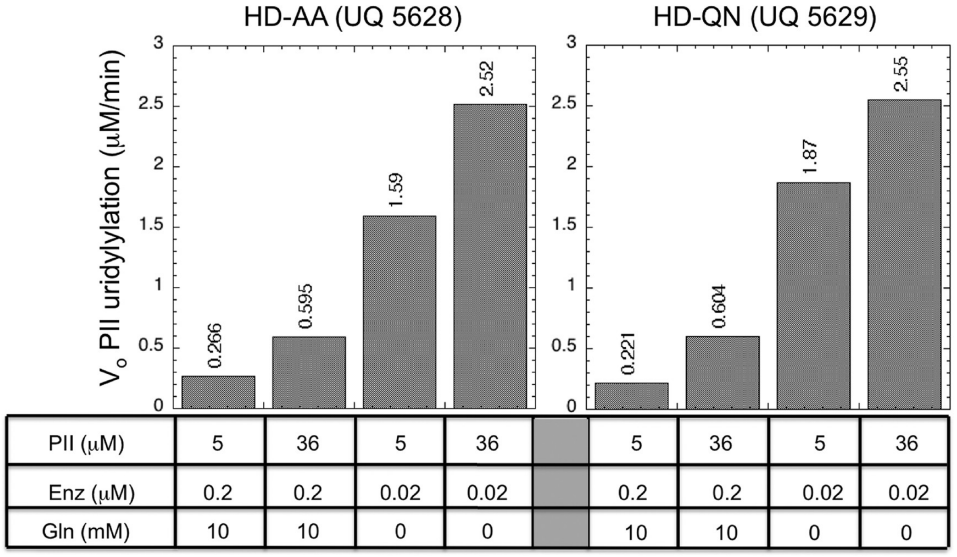

Figure 9. UT activity of the His-tagged HD-AA (UQ5628) and HD-QN (UQ5629) proteins. Initial rates of PII uridylylation were determined as described in Materials and Methods.

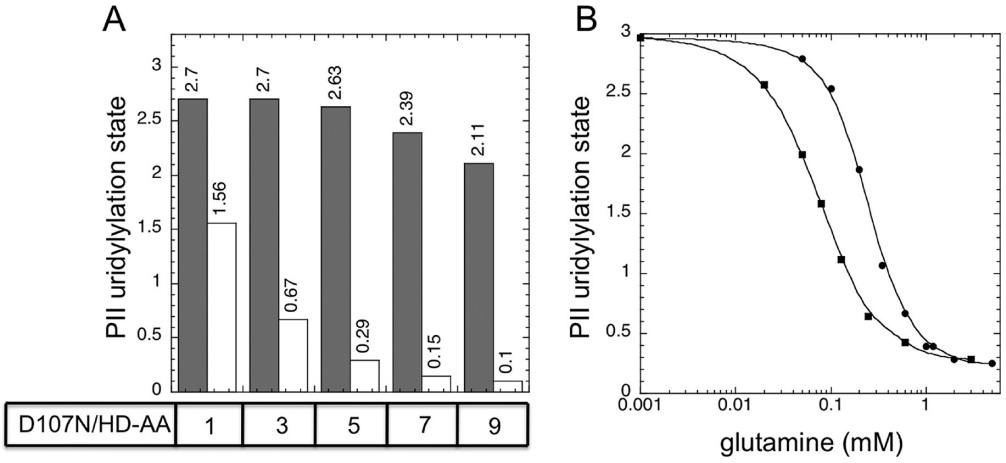

Figure 10. Reconstituted UTase/UR-PII cycles containing mixtures of the monofunctional altered HD-AA UTase/UR and D107N UTase/UR. (A) Steady-state PII uridylylation state in the absence of glutamine (filled bars) and in the presence of 10 mM glutamine (empty bars). The PII concentration was 36 μM; the His-tagged altered HD-AA UTase/UR concentration was 0.8 μM, and the altered D107N UTase/UR concentration was 0.8, 2.4, 4, 5.6, or 7.2 μM to provide the indicated enzyme ratios. (B) Glutamine signaling by reconstituted UTase/UR-PII cycles containing a combination of enzymes. The PII concentration was at 36 μM in all reaction mixtures: (■) reaction mixtures containingd 0.5 μM His-tagged HD-AA UTase/UR and 2 μM D107N UTase/UR and (●) reaction mixtures containing 0.5 μM His-tagged UTase/UR (UQ5516) and 2 μM D107N UTase/UR. The values plotted on the *y* axis were obtained in the absence of glutamine.

situation when the His-tagged bifunctional UTase/UR was used, a wide range of PII uridylylation states (~2.68 of 3.0) signaled changes in the glutamine concentration (Figure 10B). Thus, the reconstituted system comprised of monofunctional enzymes (Figure 10B) was not defective in signaling when the PII concentration was 36 μM, in contrast to the results obtained with the His-tagged bifunctional enzyme (Figure 1A). In the same experiment, we also examined the performance of a reconstituted UTase/UR-PII cycle containing the D107N and His-tagged but otherwise wild-type UTase/UR (UQ5516), using a 4:1 ratio of D107N to His-tagged UTase/UR (UQ5516). As shown in Figure 10B, a wide range of uridylylation states was also obtained (2.65 of 3.0) in this system, but other features of the glutamine response differed in the two reconstituted cycles (Figure 10B). The steepness of the response was greater in the system containing His-tagged UTase/UR (UQ5516) than in the system containing His-tagged HD-AA altered UTase/UR, and the midpoint of the glutamine response was at a higher glutamine concentration. Because the His-tagged but otherwise wild-type UTase/UR demonstrated PII substrate inhibition of UT activity, while the HD-AA enzyme did not demonstrate substrate PII inhibition of UT activity, these results may point to a role of PII substrate inhibition in determining the sensitivity of the glutamine response of the cycle.[16]

■ DISCUSSION

The addition of various "tags" to proteins to allow the use of common affinity chromatography steps in their purification has greatly advanced the analysis of numerous enzymes, but this procedure is not without risk because the tagged form of the enzyme may display altered properties. Here, we show that the addition of an N-terminal His tag to UTase/UR resulted in an altered enzyme that was defective in glutamine signaling when

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used in reconstituted covalent modification cycles. Specifically, the His-tagged enzyme displayed a defect in glutamine signaling when the PII concentration was high but displayed only a modest defect in glutamine signaling when the PII concentration was low. That is, the robustness of the covalent modification cycle to changes in PII concentration was lost as a result of the addition of a His tag to the enzyme. When the PII concentration was high, a limited range of PII uridylylation states were obtained as the glutamine concentration was varied, and these were biased toward high states of uridylylation. This defect was readily evident in side-by-side experiments where the ratio of substrate to enzyme was held constant (Figure 2); thus, it became clear that the PII concentration was the important parameter to which robustness was lost. The fortuitous observation of a defect in the robustness of the system to PII concentration allowed us to study the phenomenon and examine the underlying biochemical mechanism.

Prior studies have shown that UTase/UR consists of three functional elements: an N-terminal NT domain that catalyzes UT activity, a central HD domain that catalyzes UR activity, and a pair of tandem ACT domains at the C-terminus of the protein that is responsible for glutamine sensation.[12] ACT domains are commonly found in a tandem, paired, arrangement, so the two ACT domains may comprise a functional unit responsible for passing the glutamine signal to the other domains. We hypothesize that the domains of UTase/UR interact with and regulate their neighboring domains, and that the C-terminal glutamine-sensing ACT domains do not directly regulate the N-terminal NT domain but, rather, must pass their signal indirectly, through the central HD domain, to control the UT activity of the NT domain (Figure 7). A key to understanding the effect of PII concentration on the enzyme is the observation of PII substrate inhibition of UT activity.[15] Substrate inhibition results from the substrate binding to not only the catalytic site of an enzyme but also another site from which inhibition occurs. In the case of the UTase/UR enzyme, substrate inhibition by PII is very likely to reflect binding of PII to both the catalytic site in the N-terminal UT domain and a site in the central HD domain. PII is a product of the UR activity; thus, there is certainly a PII binding site in the HD domain. We observed that the UT activity of the His-tagged enzymes was subject to substrate inhibition by PII; thus, it seems likely that PII binds normally to the site in the HD domain in these enzymes. We also observed that altered enzymes with either a small deletion within the HD domain or two point mutations within the HD domain lacked substrate inhibition of UT activity by PII, and that the regulation of UT activity by glutamine was altered by mutations in the HD domain. These observations are consistent with binding of PII to the HD domain being the source of substrate inhibition, and with the HD domain having a role in passing the glutamine signal from the ACT domains to the UT domain.

We hypothesize that the presence of the His tag resulted in a subtle alteration of the interactions between the N-terminal nucleotidyltransferase (UTase) domain and the central HD (UR) domain. As long as PII was present at a low concentration, such that there was little occupancy of the HD domain site from which PII exerts substrate inhibition of the UT activity, glutamine regulation of UT activity of the His-tagged enzyme was nearly normal (Figure 5). However, at high PII concentrations, where PII occupied both the N-terminal catalytic site and the HD domain PII-binding site, glutamine signaling by the His-tagged enzyme was clearly defective (Figure 4). Our hypothesis to explain these observations is that, for the His-tagged enzyme, when PII bound to the HD domain it interfered with the passage of the glutamine signal to the N-terminal NT domain.

We observed that both the His-tagged (but otherwise wild-type) UTase/UR and the His-tagged HD-AA monofunctional (UTase only) enzyme could be combined with the monofunctional (UR-only) D107N altered enzyme to produce reconstituted covalent modification cycles that responded well to glutamine at the high PII concentration of 36 μM. Thus, the robustness defect of His-tagged UTase/UR could be offset simply by an increase in glutamine-regulated UR activity. This shows that the relative levels of the antagonistic activities play a key role in allowing a wide range of PII modification states in response to glutamine signaling and is consistent with theoretical studies of a covalent modification cycle.[21] We observed a defect of His-tagged UTase/UR in the regulation of UT activity by glutamine that became evident as the PII concentration was increased, but other types of defects could likely result in a similar reduction in the level of PII modification states in response to glutamine, such as simple defects in either catalytic activity. Furthermore, if an enzyme had a defect in catalysis, this could perhaps be offset by enhanced glutamine regulation of an activity so as to restore the normal relative level of the two antagonistic enzyme activities. That is, the relative levels of the UT and UR activities are likely to determine the range of modification states in response to glutamine, as predicted,[21] and a variety of mechanisms may alter or restore the natural relative levels of the activities.

Interestingly, when we compared reconstituted cycles that contained His-tagged UTase/UR and the HD-AA altered UTase/UR in combination with the D107N altered enzyme, we observed that cycles containing the His-tagged but otherwise wild-type enzyme had a steeper response to glutamine than did cycles containing the HD-AA protein (Figure 10B). Although further studies will be necessary, this finding suggests that PII substrate inhibition of UT activity may play a role in increasing the sensitivity (apparent kinetic order, Hill coefficient) of glutamine responses of the cycle. The ability of substrate inhibition to increase the sensitivity of responses of a covalent modification cycle was demonstrated by Guidi and Goldbeter.[16]

One general conclusion from our study is that the robustness of the covalent modification cycle to the concentration of its substrate depended critically on the catalytic rates of the antagonistic converter enzymes and their regulation, consistent with theory.[21] Depending on the parameters of the system, a fairly modest defect in regulation of an activity can bring about a dramatic change in the steady-state responses of the covalent modification cycle and eliminate robustness to substrate concentration. Another important observation from our study is that a loss of robustness to the concentration of the cycle substrate was manifested by a diminished range of modification states in response to the stimulatory effector. We hypothesize that this may be a common manifestation of the loss of robustness to substrate concentration.

■ ASSOCIATED CONTENT

Supporting Information

Eight additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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