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Metabolites modulate the functional state of human uridine phosphorylase I

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Abstract

Metabolic pathways in cancer cells typically become reprogrammed to support unconstrained proliferation. These abnormal metabolic states are often accompanied by accumulation of high concentrations of ATP in the cytosol, a phenomenon known as the Warburg Effect. However, how high concentrations of ATP relate to the functional state of proteins is poorly understood. Here, we comprehensively studied the influence of ATP levels on the functional state of the human enzyme, uridine phosphorylase I (hUP1), which is responsible for activating the chemotherapeutic pro-drug, 5-fluorouracil. We found that elevated levels of ATP decrease the stability of hUP1, leading to the loss of its proper folding and function. We further showed that the concentration of hUP1 exerts a critical influence on this ATP-induced destabilizing effect. In addition, we found that ATP interacts with hUP1 through a partially unfolded state and accelerates the rate of hUP1 unfolding. Interestingly, some structurally similar metabolites showed similar destabilization effects on hUP1. Our findings suggest that metabolites can alter the folding and function of a human protein, hUP1, through protein destabilization. This phenomenon may be relevant in studying the functions of proteins that exist in the specific metabolic environment of a cancer cell.

KEYWORDS

ATP, ligand-binding, protein functional state, protein stability, uridine phosphorylase I

1 | INTRODUCTION

During cancer progression, the metabolism of cancer cells usually becomes reprogrammed so as to sustain the unconstrained proliferation of these cells.[1–4] These metabolic alterations usually cause abnormal levels of metabolites. It is now clear that profiling the composition of metabolites is critical for understanding the molecular process of tumorigenesis and identifying possible treatment targets.[5–8] Among these altered metabolic pathways is the mechanism by which ATP is generated, which is found to be distinct between cancer and normal cells.[9–11] In normal cells, glucose is generally metabolized by oxidative phosphorylation in intracellular granules, an efficient catabolic process in which one molecule of glucose generates about 30 ATP molecules. In contrast, cancer cells tend to metabolize glucose via an oxygen-independent pathway in which most of the glucose taken up is not transferred into granules for glycolysis. Although the efficiency of this pathway is low, the rate of ATP generation is much higher. By accelerating the production of ATP, cancer cells usually accumulate concentrations of ATP in the cell cytosol that are 2- to 3-times

Abbreviations: 5-Fluorouracil, 5-FU; 8-Anilinonaphthalene-1-sulfonic acid, ANS; Transition state, TS; Uridine phosphorylase I, hUP1.

higher than those in normal cells. This metabolic switch associated with tumorigenesis, known as the "Warburg effect", has been studied for decades.[12–14] It is now well accepted that abnormal levels of metabolites and expression levels of metabolic enzymes are hallmarks of cancer.[15] However, little is known about how abnormal levels of metabolites such as ATP affect the function of proteins in cancer cells.

Uridine phosphorylase (EC 2.4.2.3), one of the enzymes involved in the pyrimidine nucleotide salvage pathway,[16,17] catalyzes the reversible reaction of uracil with ribose-1-phosphate in the synthesis and catabolism of uridine. The products of these reactions are further utilized for the synthesis of DNA or RNA. There are two known isoforms of uridine phosphorylase in humans: uridine phosphorylase I (hUP1) and hUP2. Of these two enzymes, hUP1, encoded by the *UPP1* gene, is generally expressed in most cells and serves as the functional protein for the uridine salvage pathway. hUP1 is composed of 310 amino acids and has a molecular weight of 33.9 kDa. The structure of this enzyme has been determined by X-ray crystallography, which shows that the functional unit is a homodimer.[18,19] It is worth mention that the homologous enzyme from *Escherichia coli* was known with similar amino acid sequence but different quaternary structure as tetramer.

Studies of hUP1 biological functions have suggested potential applications in the treatment of several cancers.[20–23] It is known that the expression level of hUP1 is usually increased in cancer cells, possibly because mutations in the tumor-suppressor protein, p53, which are common in cancer, abolish p53-mediated inhibition of hUP1 gene transcription. The high level of hUP1 in cancer cells has been exploited in the development of 5-fluorouracil (5-FU)-based chemotherapy for the treatment of various cancers,[24,25] reflecting the fact that hUP1 is the primary contributor to activation of 5-FU to produce 5-fluorouridine. This abnormal nucleotide can then be incorporated into newly synthesized RNA, resulting in destabilization of the conformation of newly synthesized RNA and disruption of its function, ultimately causing the death of cancer cells. 5-Fluorouridine can also be incorporated into newly synthesized DNA. But because fluorine is unable correctly form hydrogen bond pairs, its incorporation into DNA also damages genetic information. Thus, a higher level of hUP1 in cancer cells is expected to more effectively activate 5-FU in cancer cells than normal cells, enhancing its chemotherapeutic efficacy.

Interestingly, our recent findings suggest that ATP alters the functional state of uridine phosphorylase from *Escherichia coli*.[26] For proper biological functions, proteins usually require correct folding and structure as their functional state.[27–29] The functional state necessary for the protein to execute its activity in a physiological environment includes sufficient stability, a specified conformation, and adequate folding kinetics.[30] Ligands can sometimes interact with partially unfolded state of proteins with changed biological functions.[31] Our previous results suggested that ATP populates the partially unfolded conformation of *E. coli* uridine phosphorylase leading to the lost of enzyme activity. However, the biological role of ATP in uridine phosphorylase is unclear. With different amino sequence and quaternary structure, it is also unknown whether the functional state of human hUP1, like that of *E. coli* uridine phosphorylase, is altered by ATP. Because the expression level of hUP1 and the concentration of ATP are both elevated in cancer cells, it is also critical to understand the concentration effect of ATP or hUP1 to the functional state of hUP1.

In the current study, we comprehensively investigated the effects of ATP on the functional state of hUP1. To this end, we expressed and purified recombinant hUP1, and analyzed its protein stability and folding kinetics by pulse proteolysis. Activity assays were used to quantify the enzymatic activity of hUP1 in the presence of ATP. ANS (8-anilino-1-naphthalenesulfonic acid) binding experiments were used to assess the appearance of partially unfolded states of hUP1. Taken together, our results suggest that ATP destabilizes hUP1 and accelerates its unfolding rate. Interestingly, the concentrations of ATP and hUP1 exerted counteracting influences on the destabilization process. We further found that other, structurally similar metabolites alter the functional state of hUP1, changes that are possibly related to the effects of chemotherapy on cancer cells.

2 | MATERIALS AND METHODS

2.1 | Protein expression and purification

To optimize the expression level of hUP1 in an *E. coli* system, we synthesized the *UPP1* gene (NCBI Gene ID: 7378) based on the corresponding protein sequence (UniProt identifier: Q16831-1), adjusted to *E. coli*-optimized codons. The synthesized *UPP1* gene was then amplified by polymerase chain reaction (PCR) using the primer pair 5′-CAT GCC ATG GCT GCA ACG GGT GC-3′ and 5′-CGG GCC CGG GAT CCT TAT GC-3′ and cloned into a pET32a(+) expression vector. *E. coli* strain BL21(DE3)pLysS was subsequently transformed with the resultant plasmid and grown on ampicillin-containing LB agar plates for 12–16 hr at 37°C.

A single colony was selected and inoculated into 10 ml ampicillin-containing LB broth as a starter culture and

incubated for 12–16 hr at 37°C with constant shaking (250 rpm). This culture was then transferred into 250 ml LB broth (containing 100 μg/ml ampicillin) at a volume ratio of 1 to 100 and incubated at 37°C with shaking (250 rpm). When the culture reached an optical density at 600 nm (OD_{600}) of ~0.9, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM to induce protein expression. After incubation at 18°C (250 rpm) for 16 hr, cells were harvested by centrifugation at 3,500*g* (4°C) for 30 min. The pellet was then re-suspended in 20 mM Tris–HCl buffer (pH 8.0) containing 10 mM ethylenediaminetetraacetic acid (EDTA) and 1.0 mM dithiothreitol (DTT). Cells were subsequently disrupted by sonicating on ice, and the supernatant was collected by centrifugation at 12,000*g* for 30 min at 4°C. Because the expressed hUP1 contained a His-tag at the N-terminus, the cell lysate was first purified using poly-histidine tag affinity chromatography. Approximately 10 ml of hUP1 was applied to the column and eluted with a linear gradient of imidazole from 20 to 500 mM; fractions containing hUP1 were then pooled. Tags were removed using enterokinase, which recognizes a specific leader sequence (DDDDK) in the N-terminal region of the hUP1 construct and breaks the peptide bond after the lysine (K). Prior to the enterokinase reaction, 10 ml of cell lysate was dialyzed against 1 L of buffer solution containing 20 mM Tris–HCl (pH 7.0), 25 mM NaCl, and 1.0 mM DTT, a dialysis step required for proper function of the enzyme. This dialysis step was repeated two more times. All dialysis procedures were performed at 4°C for more than 4 hr. The total protein concentration was first measured by BCA assay, then enterokinase was added to the dialyzed sample (1 U per 50 μg protein) and incubated at 25°C for 16 hr. The enterokinase-treated sample was further purified using an SP-Sepharose fast-flow cation-exchange column. hUP1 was eluted with a linear gradient of NaCl from 25 to 500 mM, after which hUP1-containing fractions were pooled and dialyzed against 20 mM Tris–HCl (pH 7.0), 25 mM NaCl, and 1.0 mM DTT to remove excess NaCl. Dialyzed hUP1 was then concentrated, aliquoted, and stored at −80°C for subsequent experiments.

2.2 | Unfolding equilibrium

The unfolding equilibrium of hUP1 was determined using pulse proteolysis as previously described.[32,33] In short, we incubated 80 μg/ml hUP1 with 20 mM Tris–HCl (pH 8.0), 50 mM NaCl, 5.0 mM $MgCl_2$, 1.0 mM Tris (2-carboxyethyl)phosphine hydrochloride (TCEP), and a selected concentration of urea at 25°C overnight. $MgCl_2$ was added in the sample to ensure the formation of MgATP complex. Pulse proteolysis was then applied with 0.2 mg/ml thermolysin for 1 min to degrade the unfolded fraction of hUP1. Image*J* was used to quantify the band intensity of hUP1 on SDS-PAGE as the amount of remaining protein. Because the transition midpoint is a model free parameter, we used simple two-state model to fit the band intensity with varying urea concentration for determining the C_m values.

For the effect of metabolites, hUP1 was first prepared with the Tris-buffer described above as master mix. Selected metabolite was then added to the sample for the incubation of 10 minutes. Finally, urea was then added to the samples to reach the selected final concentration. After overnight incubation in room temperature, pulse proteolysis was applied to determine the fraction of folded hUP1.

2.3 | Enzymatic assay

The activity of hUP1 was measured as described previously.[34,35] In brief, the enzymatic assay was performed with reaction buffer containing 5 mM uridine, 10 mM phosphate, 10 mM Tris–HCl (pH 7.3), and 1 mM EDTA. Purified hUP1 was diluted 20 times into reaction buffer to reach the concentration of 80 μg/ml and incubated for 1 min for converting uridine into uracil. Sodium hydroxide was then added to reach 0.35 M for terminating the enzymatic reaction of hUP1. At the same time, the pH of reaction samples was adjusted to around 13 by this added sodium hydroxide. In this alkaline condition, uracil shows absorbance at 290 nm. The activity of hUP1 was then quantified by measuring the amount of the product, uracil, in each sample.

2.4 | Unfolding kinetics

The unfolding rate of hUP1 was measured as described in an earlier report.[36] Briefly, hUP1 was first prepared in 20 mM Tris–HCl (pH 7.5), 50 mM NaCl, 5.0 mM $MgCl_2$, and 1.0 mM TCEP, with or without 1 mM ATP. Next, 10.0 M urea was added to achieve a final of concentration of 1.0–4.0 M, after which the fraction of folded hUP1 remaining was monitored using pulse proteolysis. Unfolding rate constants were determined by fitting the changes in remaining protein amounts over time to a first-order rate equation. The dependence of the kinetic constant on urea concentration is described as:

$$\ln k = \ln k_{(H2O)} + \frac{m}{RT}[\text{urea}],$$

where m is the kinetic m-value of the unfolding arm.

Because dependence of unfolding constants showed a "kink" in the presence of ATP, the data were considered to represent two kinetically distinguishable steps. Two kinetic constants were used to represent these two steps, and the data were then fit to a combination of two consecutive irreversible steps. The dependence of each kinetic constant on urea concentration was modified as follows:

$$\frac{1}{k} = \frac{1}{k_1} + \frac{1}{k_2}$$

$$\ln k_i = \ln k_{i(\mathrm{H2O})} + \frac{m_i}{RT}[\mathrm{urea}] (i = 1 \text{ or } 2),$$

where k_1 and k_2 are the rate constants for the two phases, and m_i is the kinetics m-value of each unfolding phase.

2.5 | ANS experiments

To verify the presence of partially unfolded hUP1, ANS emission spectra (380–550 nm) were obtained in the presence or in the absence of 1.0 mM ATP with excitation at 380 nm. We incubated 80 μg/ml hUP1 in 20 mM Tris–HCl buffer (pH 7.5) containing 50 mM NaCl, 5.0 mM $MgCl_2$, 1.0 mM TCEP, and varying concentrations of urea (ranging from 0 to 4 M) at room temperature for overnight. Fivefold molar excess ANS was then added to the samples, and the emission intensity at 470 nm was recorded after a 2-min incubation. The fluorescence intensity at λ_{max} (470 nm) was then plotted versus the urea concentration for comparison.

2.6 | Test the effects of other metabolites to hUP1

To test the possible destabilization effects of other metabolites, we designed a simple experiment for monitoring the change of hUP1 stability in the presence of different small molecules. According to the unfolding equilibrium results, we selected a urea concentration as 1.75 M. In this condition, around 85% of the hUP1 retains the proper folded conformation. We incubated 80 μg/ml hUP1 in 20 mM Tris–HCl (pH 7.5), 50 mM NaCl, 5.0 mM $MgCl_2$, 1.0 mM TCEP, and 1.75 M urea. Different metabolites (ATP, ADP, AMP, dATP, adenosine, UTP, CTP, GTP, uridine, and phosphate) were added to achieve a final concentration of 1.0 mM. After incubation at 25°C overnight, pulse proteolysis was then applied to quantify the fraction of folded hUP1. Experiments were repeated three times for data analysis.

3 | RESULTS

To study the effects of ATP on hUP1, we first cloned the human *UPP1* gene into a pET32a(+) plasmid containing a cleavable His tag for expression of His-tagged protein in *E. coli*. Expressed proteins were first purified by polyhistidine tag affinity chromatography, after which the recombinant tags in the N-terminal leader sequence of the hUP1 peptide were removed by incubation with enterokinase. A cation exchange column was used as second purification step to obtain the purified hUP1 used for further experiments.

3.1 | ATP causes an apparent decrease in the stability and activity of hUP1

Using purified hUP1 and pulse proteolysis, we next investigated the effect of ATP on the stability of hUP1. Because hUP1 stability has never been studied using thermal dynamic experiments, we arbitrarily selected 0.8 mg/ml as the concentration of hUP1 for use in determining stability based on quantification of remaining folded protein on SDS-PAGE gels. This concentration of hUP1 (in 20 mM Tris–HCl pH 7.4, 50 mM NaCl, 1 mM TCEP) was then incubated overnight with different concentrations of urea, ranging from 0 to 4 M, and various concentration of ATP (0.5, 0.75, 1.00, and 2.00 mM). Thereafter, hUP1 was subjected to pulse proteolysis by digestion with thermolysin in the presence or absence of difference concentrations of ATP for 1 min. The residual amount of folded hUP1 was then determined by running samples on SDS-PAGE gels and quantifying band intensity by scanning densitometry. As shown in Figure 1a, the stability of hUP1 decreased with increases in the concentration of ATP. Because the value of the transition midpoint (C_m) is a model-free parameter, we used a simple two-state model to fit the data in calculating C_m. The estimated C_m values for hUP1 incubated in 0.00, 0.50, 0.75, and 1.00 mM ATP were 2.08 ± 0.02, 1.97 ± 0.01, 1.55 ± 0.01, and 0.74 ± 0.01 M, respectively. Interestingly, at an ATP concentration of 2.00 mM, hUP1 was completely digested after pulse proteolysis, even in the absence of urea. These results indicate that ATP causes an apparent decrease in the stability of hUP1. Moreover, when the concentration of ATP is high enough, hUP1 becomes susceptible to protease digestion even without the denaturant urea.

The observed increase in susceptibility to protease digestion suggests that hUP1 has lost its functional folded conformation. To determine whether this apparent change in the folded state of hUP1 is accompanied by a loss of enzymatic activity, we tested the functional

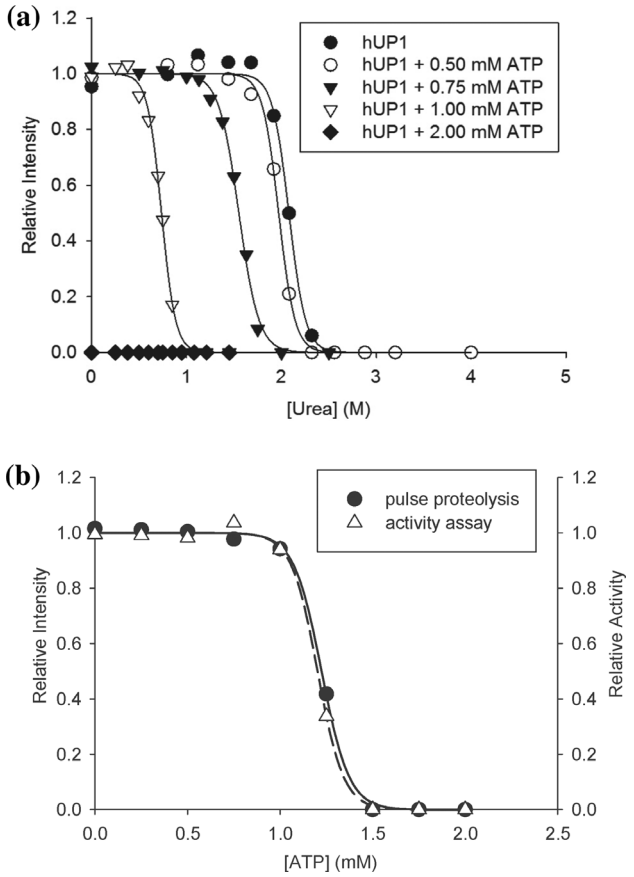

FIGURE 1 Effects of ATP on the functional state of hUP1. (a) Unfolding equilibrium of hUP1 in urea was monitored in the presence of 0 (●), 0.5 (○), 0.75 (▼), 1.00 (▽), and 2.00 (◆) mM ATP using pulse proteolysis. Relative intensity is the ratio of hUP1 band intensity remaining after pulse proteolysis to hUP1 band intensity without proteolysis. Apparent C_m values were determined by fitting relative intensities to a two-state model. (b) The influence of ATP on the functional state of hUP1 was monitored by simultaneous pulse proteolysis (●) and activity assays (Δ) in the absence of urea. hUP1 was incubated overnight at room temperature with different concentrations of ATP. Samples were then aliquoted into two sets for pulse proteolysis and activity assay. The enzymatic activity of hUP1 was quantified by measuring the amount of product, uracil, at an absorbance of 290 nm

consequences of ATP exposure using pulse proteolysis and activity assays. In these experiments, hUP1 was incubated overnight with different concentrations of ATP (0–2 mM) in the absence of denaturant, then analyzed by pulse proteolysis and activity assay. As shown in Figure 1b, hUP1 retained its folded conformation and enzyme activity at ATP concentrations less than 1 mM. However, upon increasing the concentration of ATP to 1.25 mM, only ~40% of hUP1 resisted protease digestion and retained enzyme activity. At concentrations of ATP higher than 1.5 mM, no enzymatic activity or normally folded hUP1 remained after a 1-min pulse-proteolysis procedure. Thus, hUP1 exhibited an ATP concentration-dependent increase in protease susceptibility and a corresponding decrease in enzymatic activity. These results suggest that increasing the concentration of ATP simultaneously changes the functional state of hUP1 and switches off its enzymatic activity.

3.2 | The apparent destabilizing effect of ATP also depends on the concentration of hUP1

Because the functional unit of hUP1 is a homodimer, the concentration of the protein is a factor in its stability. To understand the effect of protein concentration on the interaction between ATP and hUP1, we first measured the thermal dynamic stability of hUP1 at different protein concentrations in the absence of ATP by pulse proteolysis (Figure 2a). At hUP1 concentrations of 0.02, 0.04, 0.08, and 0.16 mg/ml, C_m values were 2.00 ± 0.02, 2.03 ± 0.02, 2.08 ± 0.02, and 2.17 ± 0.02 M, respectively. The transition midpoints increased slightly at elevated protein concentrations. Interestingly, this increase in protein stability was quite subtle, even at an eightfold increase in protein concentration, possibly because the dimer is less sensitive to changes in protein concentration. We then further determined the apparent destabilizing effect of 1 mM ATP at various concentrations of hUP1. As shown in Figure 2b, the C_m for hUP1 was also decreased in the presence of 1 mM ATP. Interestingly, this destabilizing effect of ATP was more prominent at lower concentrations of hUP1. In the presence of 1 mM ATP, C_m values at hUP1 concentrations of 0.04, 0.08, and 0.16 mg/ml were 0.36 ± 0.01, 0.74 ± 0.01, and 1.18 ± 0.01 M, respectively. Upon a further decrease in its concentration to 0.02 mg/ml, hUP1 lost its folded conformation and was completely digested after pulse proteolysis in the presence of 1 mM ATP. Thus, increasing the concentration of hUP1 actually decreases the apparent destabilizing effect of ATP.

3.3 | The effect of ATP on the unfolding kinetics of hUP1

Because ATP exerts a destabilizing effect on hUP1, it is possible that ATP also interferes with the unfolding kinetics of hUP1. To test this possibility, we measured the effects of 1 mM ATP on the unfolding kinetics of hUP1 using pulse proteolysis. As shown in Figure 3a, incubation of 0.08 mg/ml hUP1 with 3 M urea resulted in complete unfolding within 50 min. Fitting the data to a single exponential decay function yielded an unfolding

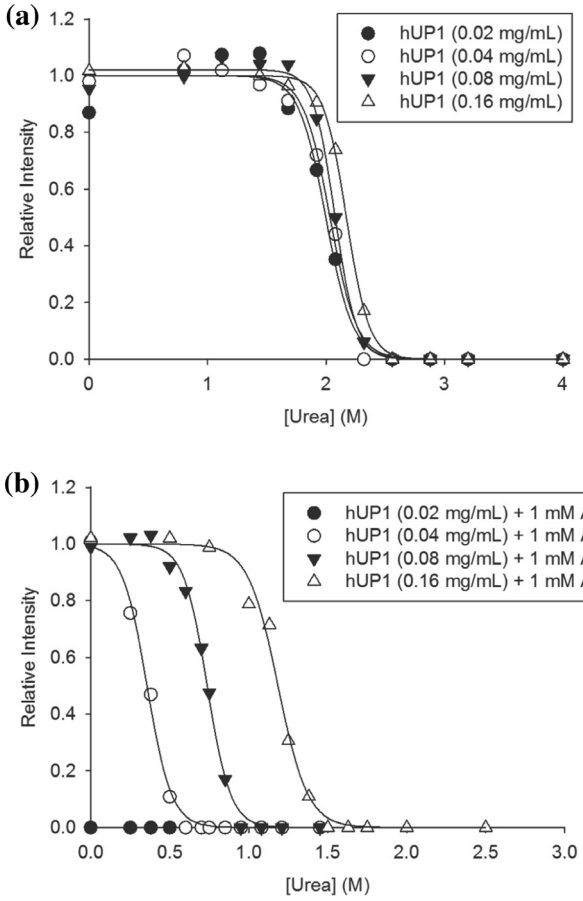

FIGURE 2 Effects of hUP1 concentration on protein stability. The stability of hUP1 was determined by pulse proteolysis in the absence (a) or presence (b) of 1.0 mM ATP. (a) Unfolding equilibrium analyses were conducted by pulse proteolysis using 0.02 (●), 0.04 (○), 0.08 (▼), and 0.16 (Δ) mg/ml hUP1. Relative intensity is the ratio of hUP1 band intensity remaining after pulse proteolysis to hUP1 band intensity without proteolysis. Apparent C_m values were determined by fitting relative intensities to a simple two-state model. (b) Pulse proteolysis was used to determine the effect of 1.0 mM ATP on the stability of hUP1 at different concentrations (●, ○, ▼, Δ). At 1 mM ATP, a low concentration of hUP1 (0.02 mg/ml) was completely digested by the protease, even in the absence of urea

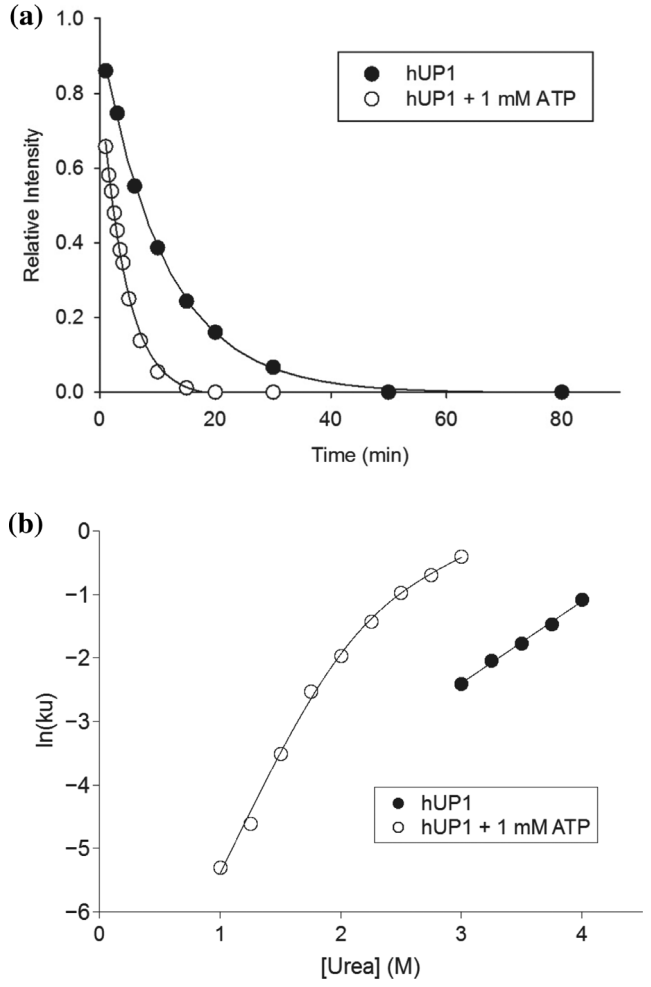

FIGURE 3 Unfolding kinetics of hUP1. (a) Unfolding of hUP1 (0.08 mg/ml) in 3.0 M urea was monitored by pulse proteolysis in the presence (○) and absence (●) of 1.0 mM ATP. Relative intensity was determined by expressing intensity at a given time point relative to the intensity of hUP1 prior to initiating the reaction (t_0). Unfolding kinetic constants were determined by fitting relative intensities to a first-order rate equation. (b) The natural logarithms of the observed unfolding rate constants (k) in the presence (○) and absence (●) of 1.0 mM ATP were plotted against the concentration of urea. The unfolding arm of hUP1 in the presence of ATP was fit to a model with two irreversible kinetic steps

rate constant of 0.09 ± 0.01 min^{-1}. We next determined the unfolding of hUP1 at the same concentration of urea in the presence of 1 mM ATP. Under these conditions, the unfolding rate increased about 7.5-fold to 0.67 ± 0.02 min^{-1}. This increase in the unfolding rate constant suggests that ATP interacts with transition states of hUP1 during the unfolding process, which was accelerated owing to a decrease in the free energy barrier.

To understand ATP-induced acceleration in the unfolding rate of hUP1, we measured the unfolding rate constant ($\ln k_u$) of hUP1 at different concentrations of urea. As shown in Figure 3b, the $\ln k_u$ of hUP1 alone linearly increased with increasing concentrations of urea, yielding a kinetic m-value of ~0.7. In the presence of 1 mM ATP, the $\ln k_u$ of hUP1 changed with increasing concentrations of urea, exhibiting two phases: at a concentration of urea lower than 2 M, the kinetic m-value of hUP1 unfolding increased to ~2.4, whereas at concentrations of urea greater than 2 M, the kinetic m-value gradually reverted back to ~0.7, a value similar to that of hUP1 alone. Changes in the kinetic m-value

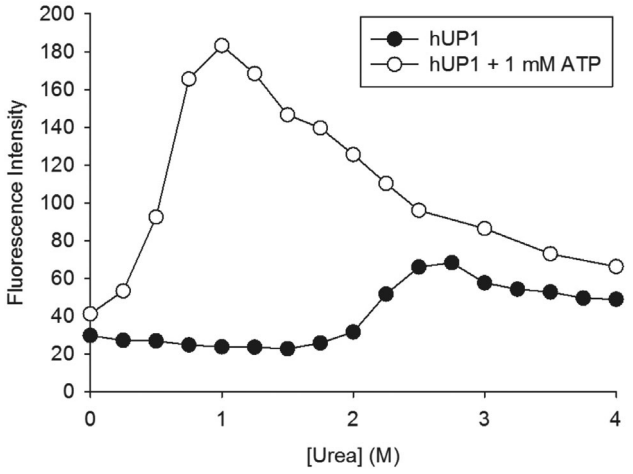

FIGURE 4 Identifying the partially unfolded conformation of hUP1. The accumulation of a partially unfolded conformation of hUP1 (0.08 mg/ml) was detected by ANS binding. The fluorescence intensity of ANS at 470 nm was measured after incubation with hUP1 in different concentrations of urea in the presence (○) or absence (●) of 1.0 mM ATP

indicate a possible switch in the rate-limiting state for hUP1 unfolding.

3.4 | ATP causes accumulation of partially unfolded states of hUP1

The increased unfolding rate constant and apparent destabilization of hUP1 in the presence of ATP suggests that ATP interacts with partially unfolded states of hUP1. Accordingly, we next determined the presence of partially unfolded conformations of hUP1 by measuring the fluorescence intensity of 8-anilinonaphthalene-1-sulfonic acid (ANS). This compound usually interacts with partially unfolded protein conformations, interactions that result in large fluorescence emission at 470 nm. Samples of hUP1 were incubated with different concentrations of urea overnight, after which ANS was added to the samples and fluorescence intensity was determined. As shown in Figure 4, hUP1 alone showed little fluorescence emission at 470 nm in the presence of ~2.5 M urea. Interestingly, incubation of hUP1 with 1 mM ATP resulted in a substantial increase in fluorescence intensity at ~1 M urea. A comparison of these results with those in Figure 1a shows that a concentration of urea of 1 M is the point at which hUP1 loses its folded structure in the presence of 1 mM ATP. A major fraction of hUP1 may be partially unfolded in this situation and thus susceptible to digestion by pulse proteolysis. These results suggest that ATP decreases the stability of hUP1 by causing the accumulation of partially unfolded, catalytically inactive states of hUP1.

3.5 | Effects of structurally similar metabolites on hUP1

Several common metabolites (Figure 5a,b) have a chemical structure similar to that of ATP. To determine whether these metabolites show destabilization effects on hUP1 similar to those of ATP, we incubated each metabolite (1 mM) with hUP1 in the presence of 1 M urea overnight at room temperature and measured the fraction of folded hUP1 by pulse proteolysis. As shown in Figure 5a, b, in the absence of metabolites ("None" in Figure 5a,b), ~70% of hUP1 remained in the folded conformation and survived pulse proteolysis treatment. In the presence of 1 mM ATP, hUP1 was completely digested after pulse proteolysis. Interestingly, ATP was not the only molecule that apparently destabilizes hUP1; ADP, CTP, and GTP also showed destabilizing effects. The effect of ADP was less than that of ATP, suggesting that the number of phosphate groups may contribute to the destabilizing effect. In contrast, dATP showed no effect on the stability of hUP1, indicating that the 2′-OH functional group on ATP may be critical for the interaction between ATP and hUP1. Among these metabolites, UTP is the only molecule that possesses a 2′-OH group and three phosphates, yet had no effect on the stability of hUP1. This result indicates that UTP may have a different mechanism of interaction with hUP1. Notable in this context, UTP possesses uridine, the known substrate of hUP1, as its base moiety. We thus carefully tested the effect of uridine on the stability of hUP1 (Figure 5c). The presence of 3 mM uridine exerted a stabilizing effect on hUP1, increasing the C_m value from 2.03 ± 0.01 to 2.24 ± 0.01 M, indicating that uridine interacts with the folded state instead of the partially unfolded state of hUP1. These results suggest that the functional state of hUP1 is modulated by the presence of various metabolites.

4 | DISCUSSION

4.1 | Increased levels of ATP alter the functional state of hUP1

We have demonstrated that the functional state of hUP1 is altered in the presence of elevated concentrations of ATP such that increasing ATP concentrations decrease the fraction of folded hUP1 and diminish enzymatic activity (Figure 1b). These results clearly suggest that the biological function of hUP1 is suppressed under conditions in which ATP is accumulated at high levels. Interestingly, cancer cells usually accumulate high concentrations of ATP in the cytosol, reflecting a metabolic shift referred to as the Warburg Effect. How much

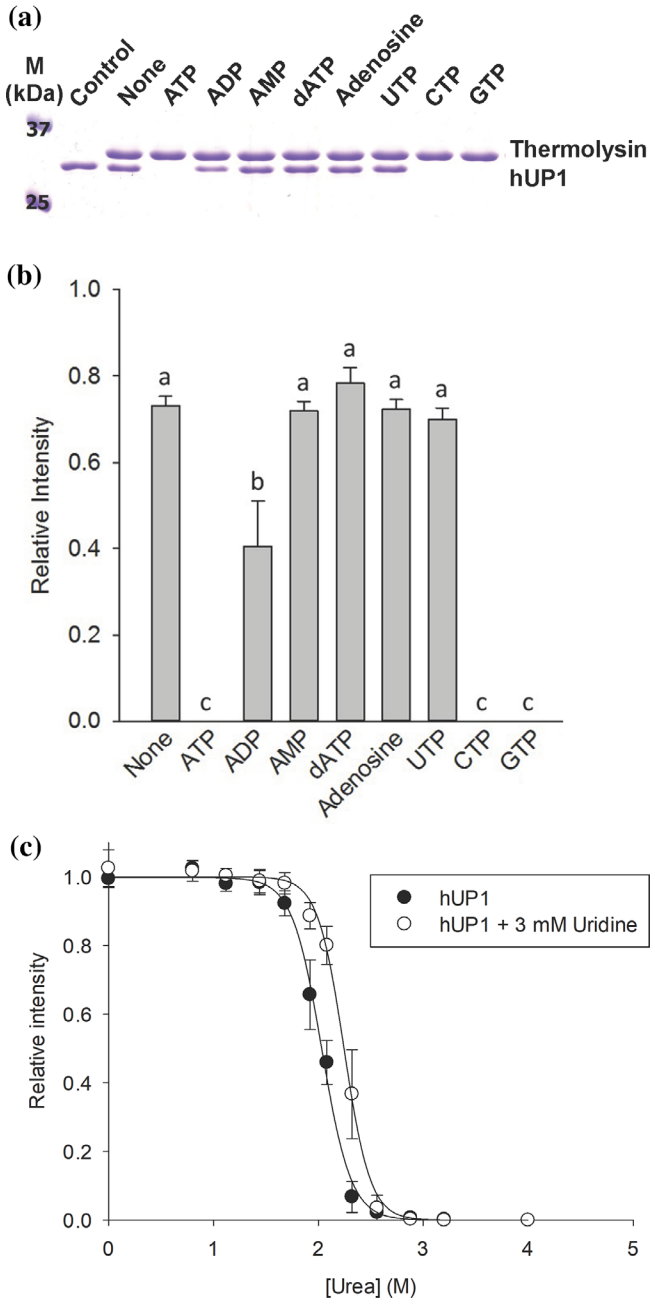

FIGURE 5 Effects of various metabolites on the stability of hUP1. (a) hUP1 (0.08 mg/ml) was unfolded for 16 hr in 1.75 M urea in the presence of various metabolites (1.0 mM) or in the absence of a metabolite (None). The amount of remaining folded protein was determined by pulse proteolysis. Undigested protein (control) is shown for comparison. (b) Relative intensity is the ratio of the band intensity of hUP1 remaining after pulse proteolysis to the band intensity of the control. The letters a, b, c indicate statistically significant differences ($p < .05$). (c) The unfolding equilibrium of hUP1 (0.08 mg/ml) was monitored using pulse proteolysis in the presence (○) and absence (●) of 3 mM uridine. The band intensity of hUP1 was normalized to that of intact hUP1 after pulse proteolysis in the absence of urea

hUP1 remains in an appropriately folded, functional state in cancer cells is now an open question. It was recently shown that directly increasing the concentration of ATP in cancer cells confers resistance to the drug, 5-FU.[37,38] Given that hUP1 is the major enzyme that activates 5-FU, the fact that increased concentrations of ATP in cancer cells destabilize the functional state of hUP1 suggests that decreases in functional hUP1 induced by elevated ATP may be responsible for the decreased activation of 5-FU. Thus, this mechanism may diminish the efficacy of 5-FU and lead to the generation of chemoresistant cancer cells. Further experiments will be necessary to quantitatively determine the fraction of folded hUP1 in cancer cells. Overall, our results collectively support the interesting concept that abnormal levels of metabolites in cancer cells alter the functional state of proteins. This phenomenon should be considered as a factor in cancer-related studies and in the development of anticancer therapies.

4.2 | The concentration of hUP1 is also a factor in the proper folding of hUP1

Another interesting observation is that the concentration of hUP1 is also a factor that contributes to the folding of this enzyme under conditions of high levels of ATP. Because the functional unit of hUP1 is a homodimer, the prediction is that increasing hUP1 protein concentration would stabilize the enzyme.[39] However, our results clearly showed that increasing the concentration of hUP1 only marginally increased hUP1 stability (Figure 2a). On the contrary, we observed that increasing the concentration of this enzyme clearly increased its stability in the presence of ATP. To explain this interesting phenomenon, we propose the thermodynamic model shown in Figure 6a. In this model, "F" denotes the folded, fully functional state of hUP1. When the concentration of hUP1 is increased, the free energy of this functional state is decreased according to the multimer effect. "I" represents an intermediate state that can interact with ATP. In the absence of ATP, the energy level of this intermediate state is always higher than that of folded and unfolded states. In other words, the observed experimental C_m value reflects the conformational change from the folded state (F) to the unfolded state (U). Further support for this model comes from ANS binding experiments performed in the absence of ATP, which showed that little fluorescence was detectable at urea concentrations from 0 to 4 M (Figure 4). The steep slope of the U state explains the slight increase in protein stability observed with elevated concentrations of hUP1. On the other hand, when 1 mM ATP is introduced into the solution, the free energy of the intermediate state is decreased. Because the intermediate state lacks a protected structure, it will be completely digested in 1-min pulse-

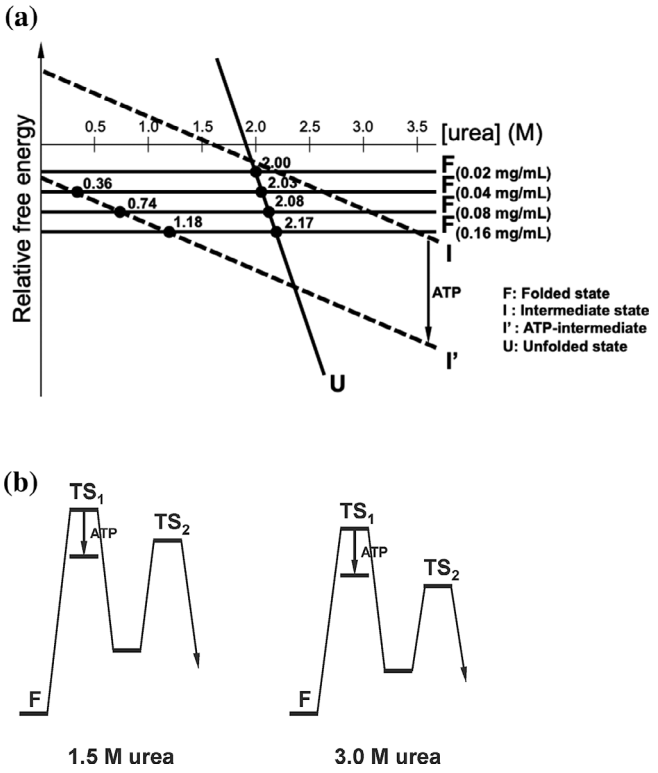

FIGURE 6 Energetic effects of ATP on the functional state of hUP1. (a) The free-energy diagram explains the accumulation of an equilibrium intermediate (I) in the presence of ATP. The four base lines, F, refer to the folded state of different concentrations of hUP1. The intersections of F and U are marked according to the C_m value of hUP1 alone, and the intersections of F and I′ are marked according to the C_m value of hUP1 in the presence of 1.0 mM ATP. (b) Unfolding kinetic models explain the increased k_u of hUP1 in the presence of ATP. The reaction energy diagram illustrates the effect of ATP on the free energy of transition state 1 (TS_1) for hUP1 unfolding. The free energies of the transition states in 1.5 and 3.0 M urea were determined using unfolding kinetics data from Figure 2b

proteolysis experiments. In this case, the experimentally determined C_m value represents the transition from the folded state (F) to the intermediate state (I′). This intermediate state may retain residual structure that can be detected by ANS binding (Figure 4). Because the intermediate state should have a solvent-assessable surface area between the folded and unfolded state, the slope of the I state should be between that of F and U states in the model. The gentle slope of the I′ state suggests that increasing the concentration of hUP1 is clearly beneficial for maintaining hUP1 in the functional state in the presence of ATP. Thus, this model can explain why protein stability increases with increasing concentrations of hUP1 in the presence of 1 mM ATP.

The effect of protein concentration on the stability of hUP1 is quite intriguing and provides some new factors that should be considered in assessments of protein functional state under physiological conditions. First, although protein concentration might show minor effects on the thermodynamic stability of a multimeric protein like hUP1, this concentration factor can become a significant contributor to protein stability that benefits the functional conformation in the presence of small molecules, such as ATP. The higher the concentration of a protein, such as hUP1, the higher its tolerance is expected to be against destabilization induced by a ligand (e.g., ATP). This potential effect, which has historically been disregarded, may be important in certain biological contexts. Second, our findings reveal that the intracellular stability of hUP1 is quite a complicated matter, requiring a consideration of the level of metabolites as well as protein concentration. Actually, this observation provides a possible explanation for the effect of ATP on hUP1, as shown in Figure 1b. The concentration of ATP in normal cells is reported to be ~1–10 mM.[40] As Figure 1b shows, most hUP1 completely loses its folded conformation and enzymatic activity at approximately 2 mM ATP, an untenable situation for a living cell. By considering the protein concentration effect, it is possible to explain how the concentration of hUP1 in cells compensates for the effect of ATP-induced destabilization. Third, cancer cells usually accumulate 2–3-fold higher ATP levels in the cytosol, but also show an increase in the concentration of hUP1. The fraction of functional hUP1 thus depends on the balance between these two factors. Further experiments will be needed to estimate the fraction of active hUP1 in cancer cells.

4.3 | ATP interacts with partially unfolded state of hUP1

It was recently reported that, at high concentrations, ATP acts as hydrotrope that contributes to the solubility of proteins in cells.[41] This finding confers on ATP a new role in addition to its conventional role as a source of biological energy—that of a chemical chaperone that helps maintain protein solubility inside cells. According to our ANS binding experiments, ATP interacts with the partially unfolded state of hUP1. Our demonstration that ATP accelerates hUP1 unfolding rate further suggests that ATP interacts with the transition state of hUP1 upon unfolding. These results suggest that ATP has the potential to interact with residual conformations of proteins. Notably, there are other proteins that show similar apparent destabilization in the presence of ATP. In addition to hUP1, *E. coli* glyceraldehyde-3-phosphate dehydrogenase and *E. coli* uridine phosphorylase interact with ATP through partially unfolded conformations.[26,42] A recent proteome-wide investigation of human cells suggested that ATP has the

potential to interact with positively charged and intrinsically disordered proteins and thereby increase their solubility.[43] hUP1 is such a positively charged protein, and its partially unfolded structure may show similar conformational features to intrinsically disordered proteins. This interesting interaction between ATP and partially unfolded proteins may be one reason why ATP helps proteins retain residual structure for proper solubility against precipitation in harsh conditions.

Our unfolding chevron-plot showed that hUP1 exhibits two kinetic *m*-values during unfolding [Figure 3b]. This change in kinetic *m*-values may indicate a switch in the unfolding rate-limiting step. The kinetic model shown in Figure 6b provides a framework for explaining this phenomenon. In the presence of a high concentration of urea (e.g., 3 M), the unfolding of hUP1 is determined by transition state 1 (TS_1). In the presence of ATP, the energy level of TS_1 is decreased, but TS_1 still remains the rate-limiting step. This is why we observed a shift in the unfolding arm with the same kinetic *m*-value in the presence of ATP. When the concentration of urea is low (e.g., 1 M), the unfolding of hUP1 is similar to the model. Without ATP, unfolding is still based on the TS_1, but in the presence of ATP, the TS_1 energy level is lower than TS_2 and unfolding rates become TS_2 dependent. Because of differences in solvent-assessable surface areas, the unfolding arm will then show a different kinetic *m*-value.

4.4 | Metabolites may alter the function of proteins in cancer cells

Based on our preliminary results, ATP is not the only molecule that interacts with hUP1 to cause apparent destabilization (Figure 5a,b). In the presence of GTP, CTP or ADP, the fraction of hUP1 in a folded conformation was lower in 1.75 M urea. In contrast, the substrate uridine increased the stability of hUP1, possibly through binding to the folded conformation (Figure 5c). These metabolites may play collaborating roles in regulating the functional state of hUP1 in cells. Changes in the levels of these metabolites, like in cancer cells, may significantly modulate the function of hUP1. A "uridine rescue" strategy was recently developed for increasing the efficacy of 5-FU in chemotherapy.[19,20,44–46] By increasing the concentration of uridine in plasma, it was found that experimental animals could tolerance higher doses of 5-FU. Moreover, this high-dose chemotherapy actually enhanced the efficiency of killing cancer cells, but not normal cells. Several drug candidates capable of increasing the concentration of uridine to support synergetic treatment of 5-FU, including 5-benzylacyclouridine (BAU) and 5-(phenylthio)acyclouridine (PTAU), have been developed.[25,47–50] However, the mechanism of uridine rescue remains unclear. One possible explanation, based on the Le Chatelier principle, is that the increased level of uridine decreases the conversion of 5-FU to 5-fluoriuridine. However, this mechanism cannot explain why cancer cells do not also exhibit enhanced tolerance to 5-FU. Our results show that a high level of ATP may decrease the fraction of functional hUP1 in cancer cells. By increasing the concentration of uridine, the functional state of hUP1 may be re-stabilized so as to support activation of 5-FU, a phenomenon that is predicted to render cancer cells more vulnerable 5-FU treatment compared with normal cells. This proposed mechanism for uridine rescue is interesting because it provides a possible new strategy for increasing the efficacy of the classical anticancer drug, 5-FU.

Cancer cells usually feature abnormal levels of metabolites in their cytosol. However, little is known about how these unusual levels of metabolites affect the functional state of proteins. Our results demonstrate that changes in the levels of metabolites might alter the functional state of proteins. These alterations may reflect the contribution of metabolite interactions with the partially unfolded state of proteins. Instead of monitoring only protein expression levels, researchers should also consider tracking changes in protein stability in cells to understand the functional state of critical enzymes. It is possible that only a fraction of a protein exhibits proper function in the context of abnormal levels of metabolites, such as in cancer cells. This observation could also drive new concepts for developing future strategies for treating cancer.

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AUTHOR CONTRIBUTIONS

Yu-Ting Huang: Data curation; investigation. **Pei-Chin Yeh:** Data curation; investigation. **Shih-Chun Lan:** Data curation. **Pei-Fen Liu:** Conceptualization; funding acquisition; project administration; resources; supervision; writing-original draft; writing-review and editing.

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