Lysine 88 Acetylation Negatively Regulates Ornithine Carbamoyltransferase Activity in Response to Nutrient Signals*

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Ornithine carbamoyltransferase (OTC) is a key enzyme in the urea cycle to detoxify ammonium produced from amino acid catabolism. OTC deficiency is an X-linked genetic disorder ranging from fatal in newborns to hyperammonemia and anorexia in adults. Through affinity purification of acetylated peptides and mass spectrometry, we identified that OTC is acetylated on lysine residues, including Lys88, which is also mutated in OTC-deficient patients. OTC acetylation was confirmed to occur under physiological conditions. Biochemical characterizations revealed that OTC Lys88 acetylation decreases the affinity for carbamoyl phosphate, one of the two OTC substrates, and the maximum velocity, whereas the $K_m$ for ornithine, the other OTC substrate, is not affected. Furthermore, Lys88 acetylation is regulated by both extracellular glucose and amino acid availability, indicating that OTC activity may be regulated by cellular metabolic status. Our results provide an example of the novel mechanism of regulating metabolic enzyme activity through protein acetylation.

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3 The abbreviations used are: OTC, ornithine carbamoyltransferase; NAM, nicotinamide; TSA, trichostatin A; IP, immunoprecipitation; PBS, phosphate-buffered saline.
bonded network that directly participates in substrate binding, indicating a critical role of Lys\textsuperscript{88} in catalysis. Furthermore, mutation of Lys\textsuperscript{88} is found in human OTC-deficient patients (20). These two lines of evidence suggest that acetylation on Lys\textsuperscript{88} may play a key role in the regulation of OTC activity. In this study, we have characterized the OTC acetylation and found that Lys\textsuperscript{88} acetylation inhibits OTC activity. Furthermore, the Lys\textsuperscript{88} acetylation is affected by cellular metabolic status, indicating a possible role of acetylation in physiological regulation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK293T and Chang liver cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (HyClone), 100 units/ml penicillin, and streptomycin (Invitrogen). Cell transfection was performed using Lipofectamine 2000 (Invitrogen) or calcium phosphate methods.

Polyclonal Antibody Generation—Pan-acetyllysine polyclonal antibodies were generated using chemically modified acetylated chicken ovalbumin as antigen. Polyclonal antibodies against acetylated OTC Lys\textsuperscript{88} were generated in rabbits using an acetylated peptide (GMIFEK(Ac)RSTRT) as antigen.

Deacetylase Inhibitors Treatment—Deacetylase inhibitor treatments were carried out by adding trichostatin A (TSA; 0.5 μM) and/or nicotinamide (NAM; 5 mM) into culture medium 16 h before harvesting; both concentrations are final concentrations in the culture medium.

Glucose/Amino Acid Treatment—Cells or transfected cells were kept in Dulbecco’s modified Eagle’s medium for 24 h before treatment. Cells were washed twice with phosphate-buffered saline and continued culture in Dulbecco’s modified Eagle’s medium (without glucose but with essential amino acids; Sigma, D5030) with the desired sup-
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implmentation of glucose or amino acids. Amino acid concentrations were the total concentrations of equimolar glutamate and aspartate. Cells were harvested after 12 h of treatment.

**Immunoprecipitation**—Cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 50 mM NaF, 1.5 mM Na$_2$VO$_4$, protease inhibitor mixture (Roche), 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). For anti-FLAG immunoprecipitation (IP), 500 µl of cell lysate was incubated with anti-FLAG M2-agarose for 4 h at 4 °C; for OTC antibody IP, lysate was incubated with anti-OTC antibody (Aviva Systems Biology; 1:500) overnight and then protein A/G beads were added, and incubation was continued for another 2 h. Beads were washed three times with lysis buffer, and the FLAG-tagged proteins were eluted by FLAG peptides (Gilson Biochemical).

**His-tagged Protein Expression and Purification**—His-tagged wild-type OTC, K88Q, and K88R proteins were expressed in *Escherichia coli* BL21(DE3). After reaching a middle exponential growing stage, 0.2 mM isopropyl-β-D-galactopyranoside was added to the bacterial culture to induce protein overexpression. Induction was carried out at 20 °C for 4 h. Expressed proteins were purified with nickel beads (GE Healthcare) as recommended by the manufacturer. Purified proteins were stored at −80 °C in 10% glycerol.

**OTC Assay**—The OTC enzyme assay was adapted from a published method (21). Briefly, 5 µl of FLAG peptide-eluted ectopic OTC-FLAG solution or 10 µl of OTC antibody-immunoprecipitated beads was added to a solution containing ornithine and triethanolamine to a final volume of 675 µl. OTC reactions were started by adding 75 µl of 150 mM carbamoyl phosphate. Final concentrations in assay of each reagent were 5 mM ornithine, 15 mM carbamoyl phosphate, and 270 mM triethanolamine, pH 7.7. After 30 min of incubation at 37 °C, reactions were stopped by adding 375 µl of phosphoric acid/sulfuric acid (3:1, v/v). Citrulline production was determined by adding 47 µl of 3% 2,3-butanedionemonoxime, boiling in the dark for 15 min, and reading absorbance at 490 nm.

**RESULTS**

**OTC Is Acetylated at Lys88**—Most reported acetylation studies are with nuclear proteins, whereas few cytoplasmic protein acetylation studies have been documented. To investigate non-nuclear protein acetylation, human liver tissue was fractionated in vivo. We found that the endogenous OTC acetylation level was significantly increased by treatment with TSA and NAM (Fig. 1B) (23). These results confirm that OTC is acetylated in vivo.

To further examine whether endogenous OTC is acetylated, Chang liver cells were treated with or without TSA plus NAM. Endogenous OTC protein was precipitated with an anti-OTC antibody, and the acetylation level of OTC was probed by anti-acetyllysine antibody. We found that the endogenous OTC acetylation level was significantly increased by treatment with TSA and NAM (Fig. 1D). These results indicate that OTC is acetylated under physiological conditions, and the acetylation of OTC is negatively regulated by deacetylases.

Our mass spectrometry data showed that OTC is possibly acetylated on two additional lysine residues, Lys46 and Lys231. A rise in total acetylation detected by anti-acetyllysine antibody is not sufficient to conclude that OTC Lys88 is acetylated. To directly detect OTC Lys88 acetylation, we raised antibody against an acetylated Lys88 OTC peptide. As shown in Fig. 1E, the site-specific antibody recognized the overexpressed wild-type OTC Lys88 acetylation, and the acetylation level of OTC Lys88 increased upon TSA plus NAM treatment. The antibody, however, could not recognize the OTC K88Q mutant, indicating that the antibody is specific for acetylated Lys88 in OTC.

![FIGURE 1. OTC is acetylated at Lys88. A, shown is a tandem mass spectrum of the acetylated OTC lysine 88-containing peptide SLGMIKFK*R. B, the acetylated Lys88 in OTC is conserved. The sequences around OTC Lys88 from different species were aligned. Conserved lysine residues corresponding to human OTC Lys88 are boxed. C, transfected OTC is acetylated. HEK293T cells were transfected with pcDNA3 vector, pcDNA3-hOTC-FLAG, and pcDNA3-FLAG-p53 followed by deacetylase inhibitor treatment. Cell lysate was immunoprecipitated with FLAG beads. The precipitated OTC-FLAG was detected by anti-FLAG antibody and anti-acetyllysine antibody (α-ACk) as indicated. NAM and TSA denote treatment with nicotinamide and triiodotironin A, respectively. D, endogenous OTC is acetylated. Chang liver cells were treated with deacetylase inhibitors. Endogenous OTC protein was immunoprecipitated with anti-OTC antibody. The acetylation level of endogenous OTC was probed by anti-acetyllysine antibody. E, OTC is acetylated on Lys88. HEK293T cells were co-transfected by pcDNA3-HOTC-FLAG and pcDNA3-HOTCK88Q-FLAG. OTC proteins were precipitated by FLAG beads. OTC Lys88 acetylation levels were detected by an antibody raised against OTC Lys88 peptide.](image-url)
Acetylation Inhibits OTC Activity

The above data demonstrate that Lys\textsuperscript{88} in OTC is indeed acetylated.

**Inhibition of Deacetylase Activity Reduces OTC Activity**—Lysine 88 has been implicated to be important for OTC catalysis (26). Therefore, acetylation of Lys\textsuperscript{88} will likely affect its enzymatic activity. We hypothesize that if OTC Lys\textsuperscript{88} acetylation regulates its enzyme activity, inhibiting deacetylases could increase the acetylation level of Lys\textsuperscript{88} and in turn, affect OTC activity. Both endogenous and overexpressed OTC activities were examined to test this hypothesis. We first examined OTC activity regulation by deacetylase inhibitors in transfected HEK293 cells. OTC was transfected into HEK293T cells, and the cells were treated with NAM or TSA. OTC protein was immunopurified, and the activity was measured. We observed that the immunoprecipitated OTC activity was inhibited in cells treated with TSA and NAM (Fig. 2A). An inverse correlation between OTC acetylation and activity strongly indicates that OTC activity is negatively regulated by acetylation. Furthermore, Chang liver cells were cultured in the absence or presence of deacetylase inhibitors, TSA and NAM. Endogenous OTC was immunoprecipitated by OTC antibody-conjugated protein A/G beads, and OTC activity was measured on beads. We found that either TSA or NAM treatment decreased endogenous OTC activity (Fig. 2B). The reduction of OTC activity by TSA and NAM indicates that endogenous OTC activity is inhibited by acetylation.

**Lys\textsuperscript{88} Acetylation Inhibits OTC Activity**—The human disease associated with the K88N mutation causes OTC deficiency, although this mutant OTC has residual activity (27). This is consistent with our finding that inhibition of deacetylases decreases OTC activity. To test the importance of Lys\textsuperscript{88} acetylation in OTC catalytic activity, we generated the K88R and K88Q mutants. The K88R mutation retains a positive charge and is thus considered as a conserved substitution. K88Q, on the other hand, abolishes the positive charge, and therefore, may mimic the effect of acetylation. Both mutants were expressed in HEK293T cells, and OTC activities were determined from immunoprecipitated protein. The K88R mutant retains substantial enzymatic activity, although it is reduced (Fig. 3A). In contrast, the K88Q mutant is essentially inactive, with <1% of the wild-type OTC activity. These results indicate that a positive charge at position 88 is critical for OTC catalytic activity. Substitution of Lys\textsuperscript{88} by a glutamine effectively abolishes OTC activity, consistent with our notion that acetylation inhibits OTC activity. We also mutated Lys\textsuperscript{46} and Lys\textsuperscript{231}, the other two acetylated lysine residues identified in our mass spectrometry data, to glutamines and found that mutation of these two lysine residues had little effect on OTC activity. These results indicate that acetylation of Lys\textsuperscript{46} and Lys\textsuperscript{231} is not directly involved in enzyme activity regulation.

It is thus of interest to determine whether Lys\textsuperscript{88} is the primary regulatory acetylation site for OTC activity. We took advantage of the fact that the K88R mutant retains substantial activity. We compared the enzymatic activity of the wild type and the K88R mutant in response to deacetylase inhibitors. As expected, treatment with TSA and NAM decreased OTC enzymatic activity with a concomitant increase in overall OTC acetylation and Lys\textsuperscript{88} acetylation (Fig. 3B). In contrast, TSA and NAM failed to inhibit the OTC K88R mutant, although overall acetylation was still increased by the deacetylase inhibitors (Fig. 3B). The increase in OTC K88R acetylation is likely due to acetylation of other lysine residues in OTC. Our data strongly indicate that acetylation of Lys\textsuperscript{88} is responsible for OTC activity inhibition in response to deacetylase inhibitors.

The three-dimensional structure of OTC suggests that a modification at Lys\textsuperscript{88} may affect OTC substrate binding and thus affect OTC activity. To further investigate the mechanism by which Lys\textsuperscript{88} acetylation reduces OTC activity, we expressed

![FIGURE 2. Inhibition of deacetylase reduces OTC activity. A, deacetylase inhibitors decrease transfected OTC activity. HEK293T cells were transfected with pcDNA3-hOTC-FLAG and treated with deacetylase inhibitors as indicated. OTC proteins were immunoprecipitated with FLAG beads and were eluted by 100 µl of FLAG peptide. OTC assay was carried out, and specific activity was normalized by OTC protein levels determined by Western blotting. Shown are mean ± S.D. of duplicate assays. The overall acetylation level and Lys\textsuperscript{88} acetylation level were assayed by anti-acetyllysine antibody or anti-acetyllysine-88 antibody. B, NAM and TSA inhibit endogenous OTC activity. Chang liver cells were treated with deacetylase inhibitors. OTC protein was immunoprecipitated with anti-OTC antibody and measure the OTC activity in protein A/G beads. Anti-hemagglutinin (HA) antibody was used as an IP control. Bars and error bars represent mean ± S.D. of triplicate assays. Specific OTC activities were normalized by the OTC protein level.](http://www.jbc.org/content/284/20/13672)
and purified wild-type OTC and the K88R and K88Q mutants from E. coli. Kinetic studies of the purified OTC show that substitution of Lys88 by either arginine or glutamine does not significantly alter the $K_m$ for ornithine, suggesting that Lys88 is not directly involved in ornithine binding (Table 1). In contrast, the K88Q mutant increased the $K_m$ of carbamoyl phosphate by 10-fold compared with the wild-type protein, whereas the K88R mutant did not significantly increase the $K_m$. These data suggest that the positive charge residue at position 88 is important for carbamoyl phosphate binding. The above observations indicate that acetylation of Lys88 in OTC may decrease its substrate binding toward carbamoyl phosphate by neutralizing the positive charge of Lys88. Given that the physiological concentration of carbamoyl phosphate is $\sim 0.1$ mM in liver cells (28, 29), the acetylated OTC would have very low activity. It is worth noting that the K88Q mutant not only altered OTC substrate binding but also dramatically decreased the maximum velocity. The OTC K88R mutant also shows a decreased $V_{\text{max}}$, although it is much less severe than the K88Q mutant. Our results support a model that OTC activity is negatively regulated by Lys88 acetylation.

Lys88 Acetylation Is Influenced by Glucose and Amino Acid Availability—OTC functions in urea cycle and amino acid catabolism, which may be affected by the availability of cellular fuel. We therefore tested the effect of glucose and amino acids on OTC acetylation. We found that lowering the glucose levels led to a decrease in Lys88 acetylation, as determined by the Lys88 acetylation-specific antibody (Fig. 4A). At the same time, OTC activity was increased by lowering glucose levels in cell culture medium (Fig. 4A), consistent with an inhibitory effect of Lys88 acetylation on OTC activity. This result suggests that an increase in Lys88 acetylation may contribute to OTC inhibition by high glucose. We also
tested the effect of amino acids (glutamate and aspartate) on OTC acetylation. Surprisingly, higher amino acid concentrations also increased OTC Lys^88 acetylation and decreased OTC activity (Fig. 4B). These observations established a direct link between OTC Lys^88 acetylation and the availability of extracellular fuels/nutrients, consistent with the notion that OTC acetylation plays a role in cellular metabolic regulation.

**DISCUSSION**

The regulatory role of protein acetylation in gene expression has been well established (30). Interestingly, many metabolic enzymes are also found to be acetylated by proteomic survey in multiple species, ranging from bacteria (31) to human. How- ever, the functional significance of acetylation in metabolic enzymes is largely unknown. Besides the mass spectrometry data of identifying acetylation, few metabolic enzymes have been functionally characterized regarding the role of acetylation in physiological regulation. One example is the acetyl-CoA synthetase, which is inhibited by lysine acetylation (15). This report on OTC provides another example that acetylation inhibits the urea cycle enzyme OTC activity by modifying a critical lysine residue in substrate binding and catalysis. Moreover, our data indicate that OTC acetylation is regulated by cellular nutrient signals, as OTC acetylation is enhanced by high glucose or amino acids.

Lysine 88 is the key acetylation site responsible for OTC inactivation. Lys^88 locates in a position important for substrate binding/catalysis. Consistently, K88Q has a much weaker affinity toward the substrate carbamoyl phosphate and is almost completely inactive (less than 1% of the wild-type activity). These observations confirm that acetylation may inhibit OTC activity. On the other hand, the K88R mutant had no effect on substrate binding and still retained ~20% catalytic activity. The moderate reduction in OTC activity of the K88R mutation could be due to the size change in the side chain upon arginine substitution, although the arginine retains the positive charge. On the basis of the mutation analysis and acetylation study, we conclude that acetylation of Lys^88 inhibits OTC catalytic activity. It is worth noting that mutation of Lys^88 has been found in human OTC deficiency disease, further supporting an important role of Lys^88 acetylation in physiological regulation.

Cells utilize glucose as the preferred energy source. When glucose is not abundant, cells shift to alternative energy sources, such as fatty acids and amino acids. Utilization of amino acids as an energy source brings two problems to the cell. First, amino acids are important building blocks for proteins, and biosynthesis of amino acids is energy-expensive; thus, amino acids would not be the preferred energy source. Second, amino acid catabolism produces ammonium, which is toxic to the body. Therefore, cells must adapt to metabolic pathways to get rid of ammonium. The urea cycle is a key pathway for ammonium metabolism. For these reasons, urea cycle enzyme activities need to adapt to different extracellular fuel availability. We found that when glucose concentration is high, OTC Lys^88 acetylation is increased, and the OTC activity is turned down (Fig. 4A). Therefore, the urea cycle activity is repressed in the

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**TABLE 1**

Kinetic parameters for OTC K88R and K88Q mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>V_{max} (μmol·mg^{-1}·min^{-1})</th>
<th>K_{m} (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type OTC</td>
<td>90.9 ± 3.5</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>OTC K88Q</td>
<td>0.6 ± 0.1</td>
<td>1.24 ± 0.20</td>
</tr>
<tr>
<td>OTC K88R</td>
<td>16.7 ± 1.9</td>
<td>0.15 ± 0.03</td>
</tr>
</tbody>
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4 W. Yu, Y. Lin, J. Yao, W. Huang, Q. Lei, Y. Xiong, S. Zhao, and K.-L. Guan, unpublished data.
and this acetylation is regulated by cellular metabolic status. We also observed that inhibition of glycolysis by 2-deoxyglucose inhibited OTC acetylation and concomitantly increased OTC activity (data not shown). Therefore, OTC activity may be regulated by cellular energy status.

OTC Lys88 acetylation is also enhanced by amino acids (Fig. 4B). Consistently, OTC activity is inhibited by high amino acid levels. However, one might expect that high amino acid levels should increase OTC activity due to an elevated demand for the urea cycle. Further studies are needed to clarify the physiological regulation of OTC acetylation in response to amino acids. Important future questions also include how glucose regulates OTC acetylation. We speculate that glucose may regulate OTC acetylation by affecting the Sirt family deacetylase. It is possible that high glucose decreases the cellular concentration of NAD, which is a cofactor for Sirt, and therefore results in a lower Sirt activity and increased OTC acetylation. In summary, our study shows that Lys88 acetylation inhibits OTC enzymatic activity and that this acetylation is regulated by cellular metabolic status and availability of nutrients.

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