Endogenous Carbamylation of Renal Medullary Proteins

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Abstract

Protein carbamylation is a post-translational modification that can occur in the presence of urea. In solution, urea is in equilibrium with ammonium cyanate, and carbamylation occurs when cyanate ions react with the amino groups of lysines, arginines, protein N-termini, as well as sulfhydryl groups of cysteines. The concentration of urea is elevated in the renal inner medulla compared with other tissues. Due to the high urea concentration, we hypothesized that carbamylation can occur endogenously within the rat inner medulla. Using immunoblotting of rat kidney cortical and medullary homogenates with a carbamyl-lysine specific antibody, we showed that carbamylation is present in a large number of inner medullary proteins. Using protein mass spectrometry (LC-MS/MS) of rat renal inner medulla, we identified 456 unique carbamylated sites in 403 proteins, including many that play important physiological roles in the renal medulla [Data can be accessed at https://helixweb.nih.gov/ESBL/Database/Carbamylation/Carbamylation_peptide_sorted.html]. We conclude that protein carbamylation occurs endogenously in the kidney, modifying many physiologically important proteins.

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Introduction

Protein carbamylation is a post-translational modification that occurs in the presence of urea. In aqueous solution, urea is in equilibrium with ammonium cyanate (200:1 molar ratio) [1]. Cyanate ion is known to carbamylate amino groups found on lysine and arginine side chains and the N-termini of proteins [2]:

\[ \text{H} - \text{N} \rightarrow \text{C} \equiv \text{O} + \text{H}_2\text{N} \rightarrow \text{H}_2\text{N} \rightarrow \text{C} - \text{NH} - \text{Protein} \]

Additionally, it reacts with sulfhydryl groups found on cysteine side chains [2,3]:

\[ \text{H} - \text{N} \rightarrow \text{C} \equiv \text{O} + \text{HS} - \text{CH}_2 - \text{CH}_2 - \text{NH}_2 \rightarrow \text{H}_2\text{N} \rightarrow \text{C} - \text{NH} - \text{Protein} \]

\[ \text{H}_2\text{N} \rightarrow \text{C} \equiv \text{S} - \text{CH}_2 - \text{CH}_2 - \text{NH}_2 \rightarrow \text{H}_2\text{N} \rightarrow \text{C} - \text{NH} - \text{Protein} \]

Several proteins have been found to be carbamylated in a variety of tissues: aquaporin-0 [4], \( \alpha B \)-crystallin [5], apoA-I [6], insulin [7], and TIMP-2 [8].

The renal inner medulla accumulates urea at high concentrations, typically greater than 500 mM during antidiuresis [9,10]. This raises the possibility that inner medullary proteins may be endogenously carbamylated due to exposure to high urea concentrations. Here, we address the question of whether there is widespread protein carbamylation in the rat renal medulla. To address this question, we used two approaches: 1) immunoblotting with a carbamyl-lysine specific antibody; and 2) large scale protein mass spectrometry of inner medullary proteins. The data showed extensive carbamylation of proteins within the renal inner medulla of rat. The mass spectrometry analysis identified 403 carbamylated proteins as well as the sites of carbamylation in their amino acid sequences.

Methods

Animals and Samples

All studies were done in male Sprague Dawley (SD) rats (250–350 g) and approved by the NHLBI Animal Care and Use Committee (Protocol Number: H-0110R2).

Animals for immunoblotting. In one set of studies, rats were water deprived for eight hours, receiving a dDAVP injection (2 nmol intraperitoneally (IP) initially and a dDAVP injection (2 nmol intramuscularly) at five hours. Kidneys were rapidly resected and dissected into the following regions: cortex (outer), cortex (inner), outer medulla (outer), outer medulla (inner), inner medulla (outer), and inner medulla (inner). In an additional set of studies, rats were either water restricted (17 ml of water/day) or water loaded (40 ml of water/day) for five days. Kidneys were rapidly resected and the whole inner medulla was removed for processing.

In all experiments, animals were euthanized by decapitation without drug treatment. Samples were homogenized (Potter-Elvehjem homogenizer on ice, 30 strokes) in a 250 mM sucrose/10 mM triethanolamine (pH 7.6) solution.

Animals for mass spectrometry. For mass spectrometry studies, rats were injected with 5 mg furosemide (IP) thirty minutes before sacrifice to lower tissue urea. These animals were neither water restricted or given a vasopressin analog. After sacrifice by decapitation, kidneys were rapidly resected and inner medullas...
were isolated. Samples were homogenized (Potter-Elvehjen homogenizer on ice, 30 passes) in a 250 mM sucrose and 10 mM triethanolamine (pH 7.6) solution.

**Immunoblotting**

Total protein content was determined with the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Samples were then diluted with sample buffer (5X Laemmli buffer) at 20% (v/v) of the entire sample volume. Proteins were resolved by SDS-PAGE (4–20% polyacrylamide gels, Criterion, Bio-Rad, Hercules, CA) and transferred electrophoretically onto nitrocellulose membranes as previously described [11]. Membranes were probed with a goat polyclonal antibody that targets carbamylated lysines in proteins (Cell Biolabs, Inc., San Diego, CA, Catalog Number: STA-077) [12]. The antibody was used at a 1:500 dilution (in Odyssey blocking buffer containing 0.1% Tween 20) overnight at 4°C. After 1-h incubation with secondary antibody (Li-Cor Biosciences 680 anti-goat immunoglobulin G; Lincoln, NE) at 1:5000 dilution, sites of antibody-antigen reaction were detected using an Odyssey infrared imager (Li-Cor).

**Mass Spectrometry**

The workflow for the mass spectrometry experiments is shown in Fig. 1. Renal inner medullary samples were passed through QIAshredder Mini Spin Columns (QIAGEN, Valencia, CA) to fragment DNA. Next, samples were dialyzed (3.5K M.W.C.O. Slide-A-Lyzer dialysis cassette, Thermo Scientific) for two hours at room temperature with urease (type III from Canavalia ensiformis, Sigma-Aldrich, St. Louis, MO) in the dialysis buffer at a concentration of 15,000 units/g. The dialysis was repeated for an additional 22 h at 4°C in the absence of urease. The total protein content of the dialyzed sample was then determined with the BCA Protein Assay Kit (Thermo Scientific). Samples were diluted in 5X Laemmli sample buffer as described above.

Each lane was equally loaded with 150 µg of protein for SDS-PAGE (4–20% polyacrylamide, Bio-Rad, Catalog Number: 345-0034). One lane was loaded with molecular weight markers (Bio-Rad, Catalog Number: 161-03775). Individual lanes were sliced with a razor blade according to the molecular weight markers to obtain 20 pieces. The gel slices were subjected to in-gel trypsin digestion, reduction, alkylation, and extraction from the gel as previously described [13].

The samples were analyzed on an Agilent 1100 nanoflow LC system (Agilent Technologies, Santa Clara, CA) connected to an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA). Spectra were matched to specific rat protein sequences using InsPecT [14], a “hybrid” algorithm that uses partial de novo sequencing combined with pattern matching. The raw data files were searched against a concatenated forward and reverse database that included the most recent RefSeq rat protein database from the National Center for Biotechnology Information (NCBI) appended with a list of common contaminants. Carbamylated peptides were identified by the presence of a +43.005814 Da modification of lysine, arginine, cysteine, or any N-terminus. The peptides identified were sorted from best to worst using the P-value parameter in InsPecT. The P-value threshold was selected to provide a one percent false positive identification rate on a peptide level.

**Pseudo Immunoblot of Water Loaded and Water Restricted Rats**

SDS-PAGE (4–20% polyacrylamide, Bio-Rad, Catalog Number: 345-0034) gels were equally loaded with 150 µg of protein. Individual lanes were sliced with a razor blade using the molecular weight marker (Bio-Rad, Catalog Number: 161-03775) as a guide to obtain 40 pieces. The gel slices were subjected to in-gel trypsin digestion, reduction, alkylation, and extraction from the gel as previously described [13].

The samples were analyzed on an Agilent 1100 nanoflow LC system connected to an LTQ Orbitrap Velos mass spectrometer as described above. Spectra were matched to specific rat protein sequences using Sequest [15]. Relative quantification of peptides was performed using QUOIL software [16]. Using the quantification data, peptide intensities for individual carbamylated peptides were summed over each of the 40 slices. To generate a heat map, we assigned values from 0–255 (8-bit gray scale) for each slice scaled over the maximum intensity found, oriented by molecular weight from top to bottom in the manner of a standard immunoblot using in-house software to generate the heat map images. Zero values (no peptides detected) were assigned a gray scale value of 5 in order to contrast with the surrounding page. Values above zero were scaled linearly.

**Bioinformatics**

Automated Bioinformatics Extractor (ABE, http://helixweb.nih.gov/ESBL/ABE/) was used to extract Gene Ontology terms from the
NCBI database as described by Hoffert et al [17]. ProMatch [18] was used to assign peptides to corresponding protein isoforms. This program is available at http://helixweb.nih.gov/ESBL/ProMatch/.

Results and Discussion

Analysis of Protein Carbamylation by Immunoblotting

Anti-carbamyl-lysine antibodies have been in use for many years [12]. We used a commercially available anti-carbamyl-lysine antibody to address whether carbamylated proteins are present in the kidneys of rats. As a preliminary confirmation of the specificity of the antibody, we incubated bovine serum albumin with 1 M urea (a concentration similar to that present normally in rat inner medulla) for two to four days and immunoblotted the samples (Figure 2). After urea incubation, the antibody recognized bovine serum albumin both as a monomer (arrow) as well as in higher molecular weight complexes. In contrast, lanes loaded with samples prepared without urea did not show these bands (Figure 2, left). This result is compatible with the manufacturer’s data pointing to specificity of this antibody for carbamylated proteins.

We used this antibody to probe immunoblots of rat renal cortex, outer medulla, and inner medulla (Figure 3). As hypothesized, many bands were seen in the inner medulla, consistent with the view that multiple carbamylated proteins are present in the inner medulla (Figure 3A). In contrast, no bands were seen in the control immunoblots in which the primary antibody was omitted (Figure 3B). Interestingly, we also observed extensive protein carbamylation in cortex and outer medulla, regions that have much lower physiological levels of urea. This finding is consistent with prior observations that proteins expressed in other organs with low local urea concentrations such as aquaporin-0 [4], αB-crystallin [5], and TIMP-2 [8] can undergo physiological carbamylation in the absence of elevated urea concentrations. Various proteins have also been shown to undergo increases in carbamylation in association with uremic levels of circulating urea [6,7,19–25].

Analysis of Protein Carbamylation by Mass Spectrometry

To identify proteins that were endogenously carbamylated in the rat inner medulla, we followed the workflow described in Figure 1. In order to remove urea from the samples prior to trypsinization, we carried out the following procedures: 1) The rats were treated with furosemide 30 minutes prior to sacrifice to reduce the urea content of the inner medulla (Diuretic conditions have been shown to markedly deplete urea from the renal medulla in rats [26]); 2) Medullary samples were dialyzed against a solution that contained no urea but did contain urease to consume urea dialyzed from the tissue; and 3) Samples were separated on SDS-PAGE using urea-free reagents and trypsinization was carried out in the gel pieces cut from these gels.

Carbamylation of Inner Medullary Proteins

Figure 3. Immunoblots of rat kidney regions with and without the -anti-carbamyl-lysine antibody. A. Immunoblot of rat kidney regions using anti-carbamyl-lysine antibody. B. Control immunoblot of rat kidney regions without primary antibody. Rats were treated with two dDAVP injections (time 0 and 5 hours). 75 ug of protein were loaded onto gels. Abbreviations: CX, cortex; OM, outer medulla; IM, inner medulla. 4–20% polyacrylamide gel.
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Figure 4. Categories of endogenous carbamylation sites found in inner medullary samples. The carbamylation sites identified are based on pooled mass spectrometry data of carbamyl sites identified in two rats. Rats were treated with furosemide 30 minutes before sacrifice but otherwise were untreated.
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Figure 2. Specificity of anti-carbamyl-lysine antibody. Bovine serum albumin (BSA, 3 mg/ml, MP Biomedicals, Solon, OH) was incubated in 1M urea (in PBS) or PBS alone for two or four days. Following incubation, samples were dialyzed using 10,000 MWCO centrifugal filter devices (Microcon, Millipore, Billerica, MA). Samples were immunoblotted as described in Methods. Arrow indicates BSA monomer. Control sample used was incubated for four days in PBS alone.
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n = 456 unique sites
The mass spectrometry analysis of rat inner medulla identified 456 unique carbamylation sites in 403 proteins. Figure 4 shows the distribution of these carbamylation sites by their locations in the proteins. The largest group (approximately one-third of the total) consists of sites that occur at the N-terminal amino acid of the proteins. Smaller fractions include carbamyl-lysines, carbamyl-arginines, and carbamyl-cysteines located in internal portions of the proteins. In addition, a significant number of peptides showed carbamylation of N-termini that are nominally in internal regions of the protein. It is unlikely that these sites are established at the time of trypsinization of the samples because of the exhaustive procedures used to deplete the samples of urea. Indeed, 56 of 127 of these N-terminal sites were carbamylated at nontrypsin sites, i.e., at sites not preceded by lysine or arginine. We propose that these internal sites are present in the intact animal as a result of the action of physiological proteolytic processes, including the protein degradation that occurs as part of normal turnover of proteins in the cell [27]. Note that many proteases in addition to trypsin, e.g. furin, cut after basic amino acids. Therefore, the remaining 71 sites may be targeted by endogenous members of this trypsin-like protease family.

Table 1. Subset of Carbamylated Proteins with Potential Physiological Relevance.

| Accession Number | Gene Symbol | Protein Name                  | Carbamylation Site |
|------------------|-------------|-------------------------------|--------------------|
| NP_036630        | Akr1b1      | aldose reductase              | *A2, C4,*H42, K33, *M13, *S23 |
| NP_077379        | Atf4        | cyclic AMP-dependent transcription factor ATF-4 | *D332, R338 |
| NP_062019        | Bsn         | bassoon                       | *L1273             |
| NP_062164        | Ca2         | carbonic anhydrase 2          | *S2                |
| NP_001012054     | Calm1       | calmodulin-like protein 3     | *A2                |
| NP_445809        | Ctnnb1      | catenin beta-1               | *A2                |
| NP_036920        | Dlg1        | disks large homolog 1         | *E561              |
| NP_00101322      | Eef1d       | elongation factor 1-delta     | *A371, *E619       |
| NP_001099307     | Eif1        | eukaryotic translation initiation factor 1 | *S2                |
| NP_001094012     | Eif2s3x     | eukaryotic translation initiation factor 2 subunit 3 | *A2                |
| NP_036686        | Eno1        | alpha-enolase isoform 1       | *L163              |
| NP_068630        | Gnb3        | guanine nucleotide-binding protein (G protein), beta-3 | C317               |
| NP_001013932     | Gnb4        | guanine nucleotide-binding protein (G protein), beta-4 | C294               |
| NP_113958        | Gnb5        | guanine nucleotide-binding protein (G protein), beta-5 | *A2                |
| NP_001244278     | Gng2        | guanine nucleotide-binding protein (G protein), gamma 2 | *A2                |
| NP_036866        | Hk1         | hexokinase-1                  | M1, *L163          |
| NP_001004082     | Hsp90ab1    | heat shock protein HSP 90-beta | E492               |
| NP_058721        | Ldha        | L-lactate dehydrogenase A chain | *A2, *A3          |
| NP_063969        | Lgal1       | galectin-1                    | *A2                |
| NP_037326        | Myh9        | myosin-9                      | *A2, *M941, *D1705 |
| NP_598267        | Ndrg2       | NDRG2                         | *A2                |
| NP_001030103     | Prkdl1      | fibrocytin-L precursor        | *V428              |
| NP_001128261     | Polr2h      | polymerase (RNA) II (DNA directed) polypeptide H | *A2                |
| NP_001178807     | Polr3b      | DNA-directed RNA polymerase III subunit RPC2 | C769               |
| NP_071943        | Ppp1cc      | serine/threonine-protein phosphatase PP1-gamma catalytic subunit | *A2                |
| NP_001178875     | Ppp1r12c    | protein phosphatase 1 regulatory subunit 12C | *S2                |
| NP_062137        | Prkar2a     | cAMP-dependent protein kinase type II-alpha regulatory subunit | *S2                |
| NP_058980        | Psmb2       | proteasome subunit beta type-2 | M1                 |
| NP_112411        | Psme5       | 26S protease regulatory subunit 8 | *A2                |
| NP_599193        | Rac1        | ras-related C3 botulinum toxin substrate 1 precursor | *P50               |
| NP_001157736     | Rnf187      | E3 ubiquitin-protein ligase RNF187 | K170               |
| NP_114467        | Ryn2        | ryanodine receptor 2 isoform 1 | R1024              |
| NP_445937        | S100a6      | S100-A6                       | *A2, C3            |
| NP_114025        | Sept9       | septin-9 isoform 1            | *S2                |
| NP_001012103     | Trim32      | E3 ubiquitin-protein ligase TRIM32 | *A2                |
| NP_001100525     | Wnt9b       | Wnt-9b precursor              | C57, K61           |

*N-terminus of peptide.
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All carbamylated peptides corresponding to Figure 4 are reported at https://helixweb.nih.gov/ESBL/Database/Carbamylation/Carbamylation_peptide_sorted.html. Table 1 shows a subset of the carbamylated proteins chosen based on potential physiological relevance. A wide variety of physiological
functions are represented. However, the functional significance of particular carbamylation sites remains unknown.

Figure 5 shows two examples of collision-induced dissociation fragmentation spectra of carbamylated peptides found in this study. Figure 5A shows an N-terminal carbamylation site in L-lactate dehydrogenase A chain (Ldha). In the spectrum, carbamylation is inferred by the presence of the added mass of a carbamyl group (minus -H) in all fragments that contain the original N-

Figure 6. Gene Ontology Cellular Component analysis reporting percent of proteins identified in various cellular structures that were found to be carbamylated. The overall total is shown as a red bar. Fraction in bars show number of carbamylated/(carbamylated+non-carbamylated) proteins. A total of 5768 proteins were detected in all samples counting proteins with both carbamylated and non-carbamylated peptides. The P values were obtained using a chi-squared test versus “Total”.

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Table 2. Identified carbamylation sites that have been previously shown to be ubiquitylated according to PhosphoSite Plus database.

| Accession Number | Gene Symbol | Protein Name                      | Carbamylation Site |
|------------------|-------------|-----------------------------------|--------------------|
| NP_112266        | Acta2       | actin, aortic smooth muscle        | K293               |
| NP_062056        | Actc1       | actin, alpha cardiac muscle 1     | K293               |
| NP_037025        | Actg2       | actin, gamma-enteric smooth muscle| K292               |
| NP_599153        | Alb         | serum albumin precursor           | K257               |
| NP_037289        | Ass1        | argininosuccinate synthase        | K165               |
| NP_001020866     | Cops5       | COP9 signalosome complex subunit 5| K54                |
| NP_00102870      | Hist1h2bc   | H2b, histone H2B                  | K58                |
| NP_001101884     | Hist1h2bm   | H2ba, histone cluster 1           | K58                |
| NP_001104597     | Hist3h2ba   | histone cluster 3                 | K58                |
| NP_001103111     | Hist3h2bb   | histone cluster 3, H2bb           | K58                |
| NP_077327        | Hspa8       | heat shock cognate 71 kDa protein | K561               |
| NP_569105        | Lcp2        | lymphocyte cytosolic protein 2    | K253               |
| NP_001157736     | Rnf187      | E3 ubiquitin-protein ligase RNF187| K170               |
| NP_741984        | Sptan1      | spectrin alpha chain, brain       | K1519              |

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terminus. Figure 5B shows carbamylation of an internal lysine present in aldose reductase (Akr1b1), a protein involved in osmotic regulation in the renal inner medulla [28]. In the spectrum, carbamylation is inferred by the presence of the added mass of a carbamyl group (minus -H) in all detectable peptide fragments that contain K33, the carbamylated lysine. Figure S1 displays six additional example spectra showing carbamylation of physiologically important proteins. In addition, the reader can view the spectra of all identified carbamylation sites online at https://helixweb.nih.gov/ESBL/Database/Carbamylation/Carbamylation_peptide_sorted.html.

Next, we asked whether carbamylation is characteristic of any particular subcellular compartment. To address this, we assigned Gene Ontology Cellular Component terms to all carbamylated and noncarbamylated proteins detected in the study. The results are summarized in Figure 6. Overall, slightly more than seven percent of identified proteins were found to be carbamylated (red bar) and all compartments are represented. We speculate that the lower fraction of lysosomal proteins that are carbamylated may be because of the low pH inside of lysosomes, which would titrate the cyanate ion (the reactive form) to cyanic acid.

What could be the physiological role of carbamylation, if any? First, like acetylation, carbamylation may alter the function of a given protein by modifying the amino acid charge, causing

Figure 7. Effects of water balance changes on carbamylation. A. Immunoblot of Water Loaded rat vs. Water Restricted rat using anti-carbamyl-lysine antibody. Animals were water restricted (17 ml of water/day) or water loaded (40 ml of water/day) for five days. B. Heat map representation of mass spectrometry data provides a pseudo immunoblot of total carbamylated proteins. Animal treatments were identical to those used in A. doi:10.1371/journal.pone.0082655.g007

Figure 8. Heat map representation of mass spectrometry data provides a pseudo immunoblot of five carbamylated proteins. For a given protein, the pseudo immunoblot shows carbamylated peptides found in each treatment group (Water Loaded vs Water Restricted) according to molecular weight and relative abundance. Molecular weights given are theoretical, based on known amino acid sequence. doi:10.1371/journal.pone.0082655.g008
positively charged moieties to become neutral [29]. This could alter the protein folding or its ability to interact with other macromolecules. Another possibility is that carbamylation of lysines, arginines, cysteines, and protein N-termini may prevent other post-translational modifications. For example, lysines undergo multiple post-translational modifications including nitration, ubiquitylation, and acetylation. These can have important consequences with regard to the function and stability of the target protein. For example, ubiquitylation is known to mark proteins for degradation either by the proteasome pathway or via lysosomes. Table 2 shows a number of carbamylation sites found in this study that have been previously identified as ubiquitylation sites (PhosphoSitePlus ubiquitylation database, http://www.phospho. site). Three example spectra showing carbamylation of peptides. Six example spectra showing carbamylation of physiologically important proteins. (TIF)

Conclusions
We conclude from these observations that protein carbamylation occurs endogenously in the kidney and is present in multiple proteins that play important physiological roles. However, contrary to the hypothesis expressed in the Introduction, protein carbamylation does not appear to be restricted to the inner medulla where urea is highest in the kidney (Fig. 3). Nor is there a striking difference in carbamylation in the inner medulla as a result of variation in water intake (Fig. 7), which is associated with striking changes in levels of tissue urea in the renal inner medulla [10]. It seems likely that circulating levels of urea, typically 4 mM in nondiuretic rats [32], is sufficient to result in carbamylation, providing an explanation for previous findings that several non-renal proteins can be carbamylated (see Introduction).

Supporting Information

Figure S1 Additional example spectra of carbamylated peptides.

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Author Contributions
Conceived and designed the experiments: JSC PCS GL CC JDH MAK. Performed the experiments: JSC PCS GL CC. Analyzed the data: JSC PCS GL CC JDH MAK. Wrote the paper: JSC PCS GL CC JDH MAK.
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