Determination of Disulfide Bond Assignment of Human Vitamin K-dependent γ-Glutamyl Carboxylase by Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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Vitamin K-dependent γ-glutamyl carboxylase is a 758 amino acid integral membrane glycoprotein that catalyzes the post-translational conversion of certain protein glutamate residues to γ-carboxyglutamate. Carboxylase has ten cysteine residues, but their form (sulfhydryl or disulfide) is largely unknown. Pudota et al. in Pudota, B. N., Miyagi, M., Hallgren, K. W., West, K. A., Crabb, J. W., Misono, K. S., and Berkner, K. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13053-13058 reported that Cys-99 and Cys-450 are the carboxylase active site residues. We determined the form of all cysteines in carboxylase using in-gel protease digestion and matrix-assisted laser desorption/ionization mass spectrometry. The spectrum of non-reduced, trypsin-digested carboxylase revealed a peak at m/z 1991.9. Only this peak disappeared in the spectrum of the reduced sample. This peak's m/z is consistent with the mass of peptide 92–100 (Cys-99) disulfide-linked with peptide 446–453 (Cys-450). To confirm its identity, the m/z 1991.9 peak was isolated by a timed ion selector as the precursor ion for further MS analysis. The fragmentation pattern exhibited two groups of triplet ions characteristic of the symmetric and asymmetric cleavage of disulfide-linked tryptic peptides containing Cys-99 and Cys-450. Mutation of either Cys-99 or Cys-450 caused loss of enzymatic activity.

We created a carboxylase variant with both C598A and C700A, leaving Cys-450 as the only remaining cysteine residue in the 60-kDa fragment created by limited trypsin digestion. Analysis of this fully active mutant enzyme showed a 30- and the 60-kDa fragment were joined under non-reducing conditions, thus confirming Cys-450 participates in a disulfide bond. Our results indicate that Cys-99 and Cys-450 form the only disulfide bond in carboxylase.

The vitamin K-dependent carboxylase is an integral membrane glycoprotein that catalyzes the post-translational modification of specific glutamic acid residues to γ-carboxyglutamic acid (Gla)1 (1, 2). The carboxylation reaction occurs in the lumen of the ER (3, 4) and uses the substrates carbon dioxide, oxygen, and vitamin K hydroquinone. During the process of carboxylation, the γ-proton of the glutamic acid is abstracted, followed by the addition of carbon dioxide (5). Simultaneous with carboxylation, the vitamin K hydroquinone is converted to vitamin K epoxide, which is converted back to vitamin K by the enzyme epoxide reductase. The formation of vitamin K epoxide has sometimes been called an epoxidation reaction. Gla modification is critical for the function of more than a dozen proteins involved in blood coagulation and calcium homeostasis (6, 7).

The importance of vitamin K-dependent proteins may be even greater than previously thought, as evidenced by the discovery of growth-arrest protein gas-6 (8), and the very recent identification of four putative vitamin K-dependent membrane Gla proteins PRGP1, PRGP2, TMG3, and TMG4 (9, 10).

There are ten cysteine residues in the human carboxylase molecule. Our work on the topology of the carboxylase predicts that of these ten cysteines, two are located in the cytoplasm, three are buried in the ER membrane, and five are found in the lumen of the ER (11). Sulfhydryl groups and disulfide bonds are important for both the structure and function of proteins (12–15). For example, the natural abundance of cysteine is 1.2%, but these residues constitute 5.6% of enzyme catalytic sites (16). Therefore, identification of free cysteine residues or those involved in disulfide bond formation can give valuable information about the structure and function of proteins.

Several studies have implicated cysteine in the function of carboxylase. Chemical modification of carboxylase by sulfhydryl-reactive reagents suggests that cysteine residues are important for the carboxylation reaction (17–21). Based on a non-enzymatic chemical model, Paul Dowd et al. (22) developed a “base strength amplification mechanism” for carboxylation. They proposed that two free cysteines are involved in the active site of carboxylase. Recently, Pudota et al. (21) analyzed the catalytically important cysteine residues of the carboxylase by modifying free cysteines with the radiolabeled sulfhydryl-reactive reagent 14C-NEM. These authors reported that cysteine residues 99 and 450 are the active site residues of carboxylase (21). In contrast to the multiple studies on the importance of free cysteines in carboxylase, the only information about disulfide bridges in the structure of the carboxylase is the study by TMG, transmembrane γ-carboxyglutamic acid proteins; CHAPS, 3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate; PC, 1,2-dioleoyl-sn-glycerol-3-phosphocholine; NEM, N-ethylmaleimide; TCEP, tris(2-carboxyethyl)phosphine; MOPS, 3-(N-morpholino)propanesulfonic acid; CID, collisionally induced dissociation; LC-ESMS, liquid chromatography electrospray mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; ESI-MS, electrospray ionization mass spectrometry.

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Wu et al. (23). This study demonstrated that limited trypsin digestion of carboxylase at residues 349 and 351 results in a 30 kDa amino-terminal fragment disulfide linked to a 60 kDa carboxyl-terminal fragment.

Although mass spectrometry has been used to characterize soluble proteins, its application to integral membrane proteins has lagged because of their unique characteristics (24, 25). Integral membrane proteins represent around 30% of all proteins and play important roles in various cellular processes including signal transduction, cell adhesion, ion transport, endocytosis, and enzymatic reactions (26–27). The hydrophobic nature of the integral membrane proteins requires the use of detergents for solubilization and stability (28), but most detergents are not compatible with mass spectrometric analysis. Therefore, new approaches for the application of mass spectrometry to characterize and identify membrane proteins are being actively pursued (25, 29, 30). Among recent studies, van Montfort et al. (30) reported an improved in-gel approach to generate peptide maps of integral membrane proteins for MALDI-MS.

In this study, we have used in-gel protease digestion and MALDI-TOF MS/MS to identify the disulfide linkage of the human vitamin K-dependent carboxylase. Our results indicate that a disulfide bond joins cysteine residues 99 and 450. Mutation of either of these two cysteine significantly decreases the carboxylase activity. This is expected if the structure of the enzyme is compromised by the lack of the disulfide bond.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were reagent grade. CHAPS, n-octyl-β-D-glucopyranoside, α-cyano-4-hydroxysuccinimide acid, and N-ethylmaleimide (NEM) were obtained from Sigma (St. Louis, MO). 1,2-Diisobutyl-N-glucopyranosyl (PC) was from Avanti (Alabaster, AL). H-β-The-Pre-Arg-chloromethylketone and FLEEL were from Bachem (King of Prussia, PA). NaH14CO3 (specific activity, 54 mCi/mmol) was from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Tris(2-carboxyethyl)phosphine (TCEP) hydrochloride was from Molecular Probes (Eugene, OR). Vitamin K1 was from Abbott Laboratories (Chicago, IL). PNGase F (500,000 units/ml) and all the restriction enzymes were from New England Biolabs (Beverly, MA). Aprotinin and sequencing grade modified trypsin and chymotrypsin were from Roche (Indianapolis, IN). The BacVector-3000 kit for insect cell transfection was from Novagen (Madison, WI). Coomassie Blue R-250, protein standard markers, and SDS-PAGE ready gel were from Bio-Rad. SP-Sepharose was from Amershon Biochemicals (Madison, WI). The carboxylase activity assay was conducted with the "Megaprimer" method of PCR mutagenesis (31, 32). Wild-type human carboxylase cDNA with a FLAG tag (DYKDDDDK) at the amino terminus and a HPC4 tag (EDQVD-C) at the carboxyl terminus (33) was used as template DNA for PCR. Mutations were screened by restriction digestion, verified by sequencing, and expressed carboxylase was purified by affinity chromatography using the anti-HPC4 antibody-coupled Sepharose resin as described (34). Further expression and purification of human carboxylase in insect cells was performed as previously described (23). A pilot reaction was run for each batch of trypsin.

**Limited Trypsinization of Carboxylase**—Limited trypsin digestions of carboxylase in the presence of the protease were performed as previously described (33). This reaction was started by the addition of 10 μl of an ice-cold mix of NaH14CO3 and vitamin K hydroquinone; the reaction was stopped by adding 20 μl of 5% trichloroacetic acid, and the amount of14CO2 was determined in a scintillation counter. The mass spectrometric analysis of the fragments of either angiotensin I or adrenocorticotropic hormone for site-directed mutagenesis were synthesized by Invitrogen Life Tech;

**Expression and Purification of Human Carboxylase in Insect Cells**—Wild-type or cysteine mutant carboxylase cDNA engineered in the expression vector pVL1392 was used as template DNA for PCR. Mutations were screened by restriction digestion, verified by sequencing, and expressed carboxylase was purified by affinity chromatography using the anti-HPC4 antibody-coupled Sepharose resin as described (34). Further expression and purification of human carboxylase in insect cells was performed as previously described (23). A pilot reaction was run for each batch of trypsin.

**Determination of Disulfide Bond in Carboxylase**—The carboxylase activity assay was performed in a total volume of 125 μl containing 25 mM MOPS, pH 7.5, 500 mM NaCl, 0.8 M (NH4)2SO4, 0.12% PC, 0.26% CHAPS, 4 μg proFX, 5 μCi of NaH14CO3 (specific activity, 54 mCi/mmol), 222 μM vitamin K hydroquinone, and 1.25 mM FLEEL. The reaction was started by the addition of 10 μl of an ice-cold mix of NaH14CO3 and vitamin K hydroquinone; the reaction mix was immediately transferred to a 20 °C water bath and incubated for 30 min. The reaction was terminated by the addition of 1 ml of 5% trichloroacetic acid, and the amount of14CO2 was determined in a scintillation counter. The mass spectrometric analysis of the fragments of either angiotensin I or adrenocorticotropic hormone for site-directed mutagenesis were synthesized by Invitrogen Life Tech;

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and carboxylase to determine the optimal conditions for limited cleavage. Cleavage was accomplished on ice in the presence of 5 μM proFIX. Trypsin cleavage was stopped by the addition of H-μ-Phe-Pro-Arg chloromethylketone and Aprotinin to a final concentration of 1.6 μM. Samples were subjected to a reducing and non-reducing gradient (4–20%) SDS-PAGE, and the protein bands were made visible through silver staining.

## RESULTS

**Peptide Mass Fingerprint of Human Vitamin K-dependent Carboxylase by MALDI-TOF MS**—Freshly purified carboxylase (37) was deglycosylated, fractionated by SDS-PAGE, and digested in-gel (30). Fig. 1 shows a typical MALDI-TOF mass spectrum of the trypsin-digested non-reduced carboxylase. The MS-Fit search program (38), a part of “Protein Prospector” Version 4.0.4 (available at prospector.ucsf.edu), was used to identify the mass peaks in the “NCBI n5.28.2003” data base. The results of the search are shown in Table I. Cysteine-containing peptides are marked by an asterisk in Fig. 1. A diagram of the sequence coverage of carboxylase by MALDI-TOF MS is shown in Fig. 2. For trypsin digestion, 38.8% of the expected peptides were recovered; this coverage could be increased to 41.9% by re-extraction of the gel pieces after the supernatant had been removed.

There are three peptide peaks (Table I) at m/z 2373.2, 2433.3, and 2690.4 corresponding to residues 458–476, 625–647, and 623–647 that differ by 1 unit from the expected trypsin-digested fragments. Each of these peptides has a consensus sequence for N-glycosylation. Because deglycosylation by PNGase F converts an asparagine residue to an aspartic acid residue, the 1-unit mass shift is expected. This prediction was confirmed by MS/MS sequencing of the tryptic peptides and deglycosylation of carboxylase in the presence of 18O-labeled water (data not shown).

To increase the sequence coverage, limited chymotrypsin digestion of carboxylase was performed. Chymotryptic digestion alone covers 30.5% of the carboxylase sequence, some of which overlaps with the sequence recovered by trypsin digestion (Fig. 2). Total sequence coverage with trypsin and chymotrypsin was 62.3% of the entire carboxylase. Higher sequence coverage is difficult to obtain because the hydrophobic peptide region and the membrane-spanning segments are either not readily accessible to proteolytic enzymes, and/or are difficult to extract from the gel pieces. As shown in Fig. 2, peptides containing all of the ten cysteines were recovered.

**Disulfide-linked Peptides of the Carboxylase Molecule**—The program MS-Fit in Protein Prospector was used to search for possible disulfide-linked peptides in both the trypsin and chymotrypsin digestions of the non-reduced carboxylase samples.
The candidate peaks were then compared with the peptides from a MALDI-TOF mass spectrum of the reduced sample. A peptide ion of m/z at 1991.9 (Fig. 3A) from the trypsin-digested non-reduced sample matches the expected size of a peptide consisting of residues 92–100 (m/z at 1053.5) disulfide-linked to residues 446–453 (m/z at 941.4). As would be expected if this peak represented a disulfide-linked peptide, it was not present in the spectrum of the reduced sample (Fig. 3B). This is the only candidate disulfide-linked peptide that disappears in the reduced sample. Therefore, it appears to be the only disulfide bond in the carboxylase because peptides containing all of the ten cysteines were identified (Fig. 2). Small signals of the reduced forms of peptides 92–100 (m/z at 1053.5) and 446–453 (m/z at 941.4) were also observed in the non-reduced trypsin-digested carboxylase sample (Table I). This is the result of MALDI-induced cleavage of the disulfide bond, as observed by other groups (39–43). In addition, there is a small amount of free cysteine 99 and 450 in the non-reduced sample vide infra.

MS/MS Analysis of Peptide m/z at 1991.9—We isolated the peptide consisting of residues 92–100 disulfide-linked to residues 446–453 at m/z 1991.9 by a timed ion selector as the precursor ion for further MS analysis (Fig. 4). Fragmentation spectrum revealed two sets of strong triplet signals at m/z 1019.5, 1053.5, and 1085.4, as well as 907.4, 941.4, and 973.4. These ion peaks correspond to the symmetric and asymmetric cleavage of the disulfide bond linking the peptides (40, 42, 44, 45). The intensities of peptide ions m/z at 973.4 and 1019.5 are relatively higher than the others, indicating that fragmentation of the disulfide-linked heteropeptide favors asymmetric cleavage with retention of the two sulfurs on one peptide fragment (peptide 446–453 in this case); this agrees with the observations of Jones et al. (40).

Further proof that peptides 92–100 and 446–453 are disulfide bonded is provided by the presence of additional peaks expected from the fragmentation of the parent ion. The peak at m/z 1835.7 is expected from the loss of one arginine from the intact disulfide-linked parent ion. The peaks at m/z 1600.7 and 1315.6 correspond to the fragmentation of disulfide-linked peptide 92–100 between residues 94 and 95 (m/z 1600.7) and 97 and 98 (m/z 1315.6) (Fig. 4B). Finally, two ion fragments predicted from peptide 92–100 were observed at m/z 662.3 and 889.4.

Identification of NEM-modified Cysteine Residues of the Carboxylase—NEM-modified and non-modified carboxylase were digested with trypsin or chymotrypsin and analyzed by MALDI-TOF MS to detect modified cysteines. Modification of cysteine by NEM will cause a mass increase of 125.1 Da. The major peaks representing NEM-modified peptides were peptide 319–326 containing Cys-323 (m/z 1090.5) and peptide 335–346 containing Cys-343 (m/z at 1374.6) from the tryptic digestion (Fig. 5); and peptide 136–143 containing Cys-139 (m/z 962.5) and peptide 310–319 containing Cys-311 (m/z 1387.6) from the chymotrypsin-digested sample (Fig. 6). In the trypsin-digested non-reduced sample, we also found trace amounts of NEM-modified peptides containing Cys-99 and Cys-450, as indicated by small signals at m/z 1178.5 and 1066.5 (Fig. 7, B and D). However, the major signal representing cysteine 99 and 450 was the disulfide-linked peptides containing 92–100 and 446–453 (m/z 1991.9). Furthermore, as described above, after reduction, this peak was eliminated. This indicates that the majority of cysteine residues 99 and 450 of carboxylase are disulfide bonded, and that cysteine residues 323, 343, 139, and 311 were the major residues modified by NEM.

Cysteine Mutants of the Carboxylase—We used site-directed mutagenesis to mutate cysteine residues of carboxylase to serine or alanine. Mutant proteins (Table II) were expressed and affinity purified from insect cells. The specific activity of each enzyme was determined (37) and compared with that of the wild type. In agreement with Pudota et al. (21), mutation of
either cysteine 99 or 450 results in a significant decrease of carboxylase activity. In contrast to their results, however, we found that C288S had only 56% activity, while they reported that this mutation had no effect on activity. Another discrepancy with their result is that we found that C343S had normal activity, while they found that its activity was significantly reduced compared with wild-type enzyme (35); all the other mutants were fully active.

**Limited Trypsin Digestion of Cysteine Mutant Carboxylase**—

Previous work from our laboratory showed that limited trypsin digestion of carboxylase results in a disulfide-linked 30 kDa amino-terminal and 60 kDa carboxyl-terminal fragments (23). Fig. 8 shows the results of limited trypsin digestion of cysteine mutant carboxylase C450A and C598A/C700A. Purified C450A (Fig. 8A, lane 2) is more degraded than the wild-type enzyme (Fig. 8A, lane 1) and instead of the expected 30- and 60-kDa...
fragments, limited trypsin digestion results in further degradation (Fig. 8A, lane 3); a similar result was observed for C99S (data not shown). While this is not direct evidence that cysteine 450 and 99 are disulfide-bonded, degradation might be expected if elimination of a disulfide bond reduced the structural stability of the enzyme. Direct evidence that at least cysteine 450 is part of a disulfide bond is provided by limited trypsin digestion of the fully active mutant carboxylase C598A/C700A. The 30- and 60-kDa fragments are still connected by a disulfide bond (Fig. 8B, lane 5) when analyzed by SDS-PAGE. When the sample is reduced, however, the 30- and 60-kDa tryptic fragments appear (Fig. 8B, lane 3). Since the only remaining cysteine residue in the 60-kDa fragment of the C598A/C700A mutant carboxylase is cysteine 450 (Fig. 9), it must be linked by a disulfide bond to a cysteine in the 30-kDa fragment.

**DISCUSSION**

Exploring the structure and function roles of cysteine residues in carboxylase is important for several reasons. First, several laboratories have shown that sulfhydryl reactive reagents inhibit the carboxylase activity, suggesting that free cysteine residues are important for the carboxylation reaction (17–21). Furthermore, based upon this inactivation of carboxylase by sulfhydryl reactive reagents and a non-enzymatic chemical model, a detailed mechanism for carboxylation that utilized two cysteines as active site residues was proposed (22).

In addition to their possible role as active site residues, the number and connectivity of the disulfide bonds is important for understanding the three-dimensional structure of an enzyme and provides invaluable information for enzyme function. In this report, we used in-gel protease digestion and MALDI-TOF mass spectrometry to determine the disulfide assignment in human carboxylase. Disulfide-linked tryptic peptides 92–100 and 446–453 of carboxylase were found in the MALDI-TOF mass spectrometry of the non-reduced sample (m/z 1991.9) but not in the reduced sample (Fig. 3), suggesting that cysteines 99 and 450 are involved in disulfide bond formation in the carboxylase molecule.

To further confirm the disulfide linkage between tryptic peptides 92–100 and 446–453, the precursor ion detected at m/z 1991.9 was subjected to MS/MS for CID fragmentation of the disulfide bond. It has been reported that cleavage of a disulfide bond can produce a dehydroalanine, a cysteine, and a thiocteine through β-elimination (46, 47). The presence of these three amino acids has also been observed in MALDI-TOF mass spectrometry (40, 42, 44, 45), and was attributed to asymmetric and symmetric cleavage of the disulfide bond of the disulfide-linked peptides during fragmentation. The existence of these triplet peptide ions differing in a single sulfur atom is considered to be characteristic of the fragmentation of a disulfide-linked peptide in MALDI-MS. Fig. 4 shows the MS/MS spectrum of the precursor ion at m/z 1991.9; two groups of strong triplet ion signals appear with a mass difference of a single sulfur atom.

**Table II**

Comparison of the specific activity of cysteine mutant carboxylase and the wild-type carboxylase

| Carboxylase | Specific activity (mean ± S.D., n = 3) | Percent activity |
|------------|--------------------------------------|-----------------|
| WT         | 1192 ± 17                            | 100             |
| C99S       | 6.7 ± 0.63                           | 0.56            |
| C134W      | 1196 ± 11                            | 99.5            |
| C133S      | 1061 ± 13                            | 89.0            |
| C288S      | 867 ± 13                             | 56.0            |
| C311S      | 1178 ± 21                            | 98.8            |
| C323S      | 1144 ± 24                            | 96              |
| C343S      | 1240 ± 31                            | 104             |
| C450A      | 7.6 ± 0.26                           | 0.64            |
| C598A/C700A| 1109 ± 29                            | 93.0            |

Fig. 7. Comparison of the MALDI-TOF mass spectra of the trypsin digestion of carboxylase and NEM-modified carboxylase (NEM modification of Cys-99 and Cys-450). Panel A, unmodified carboxylase spectrum from m/z 1178 to 1193. Panel B, NEM-modified carboxylase spectrum from m/z 1061.0 to 1069.0. Panel C, unmodified carboxylase spectrum from m/z 1061.0 to 1069.0. Peptide ions appear at 1178.56 (B) and 1066.52 (D), corresponding to the NEM-labeled peptide containing cysteine 99 and 450, respectively. Inset (panel D) magnifies the base line because the abundance of NEM-modified cysteine 450 is low due to its involvement in disulfide bridging.
Our result that cysteine residues 99 and 450 of carboxylase are joined by a disulfide bond differs from the result of Pudota et al. (21). These results are consistent with the notion that the 30- and 60-kDa tryptic fragments observed in wild-type carboxylase are a result of structural modification rather than inhibition of enzyme catalysis (21). To determine why our results differ from those of Pudota et al., we repeated their NEM experiments (21). Our results show that only trace amounts of cysteine 99 and 450 containing peptides were modified by NEM (Fig. 7), while the majority of these two peptides are linked by disulfide bonds (Fig. 3). This result is consistent with our previous result of limited trypsin digestion of carboxylase, which shows that the majority of the 30- and 60-kDa tryptic fragments are joined by disulfide bond(s) (23). Due to the high sensitivity of MALDI-TOF mass spectrometry, we propose that the existence of trace amounts of NEM-modified carboxylase residues 99 and 450 and tryptic peptide sequences in our experiments, and probably in those of Pudota et al. (21), is due to the NEM modification of small amounts of the non-disulfide-bonded proteins. In some cases, however, they found that cysteine 99 was modified by both NEM and iodoacetamide, suggesting that cysteine 99 existed as two different forms in the purified enzyme.

Although Pudota et al. (21) did not report the relative abundance of the ions they monitored, we assume the signals attributed to NEM-modified cysteine residues 99 and 450 were substantially above background. It is possible that the seemingly larger fraction of NEM-modified cysteine residues 99 and 450 observed, compared with our own results, is also due, in part, to the different analysis methods employed. For complex mixtures, the dynamic range of ESI coupled to LC separation surpasses that of direct MALDI. The peptides in question may have eluted from the column during a retention window free of more abundant peptides, in which case a greater fraction of the peptide population would appear ionized, perhaps leading to some distortion in the relative abundance. It is also important to note that peptide assignments should be confirmed by MS/MS sequence information, as we have done in this study.

Site-directed mutagenesis of the ten cysteine residues of carboxylase (Table II) shows that only cysteines 99 and 450 have a significant effect on enzymatic activity. Although modification of cysteines 139, 311, 323, and 343 by NEM can apparently inhibit carboxylase activity, mutations of these cysteine residues have little or no effect on the enzyme activity. We suspect that the reason that NEM inactivates carboxylase is a result of structural modification rather than inhibition of an active site cysteine residue(s). We also have different results from those of Pudota et al. (21, 35) regarding mutations of cysteines 99 and 450 in carboxylase, we replaced them with either serine or alanine. In agreement with Pudota et al. (21), mutant enzymes C450A and C99S have significantly decreased carboxylase activity (Table II). We propose that the loss of activity is due to the involvement of these two cysteines in the active site residues of carboxylase. They used radioactive [14C]NEM to modify the free cysteine residues of native carboxylase, quenched the reaction with dithiothreitol, denatured the NEM-modified carboxylase with urea, and modified the remaining cysteines with iodoacetamide. The [14C]NEM and iodoacetamide-modified carboxylase was then digested with trypsin and analyzed by LC-ESMS. Their results suggest that only cysteine residues 99 and 450 are modified by NEM (21). Since mutations of cysteines 99 and 450 dramatically decreased carboxylase activity, they concluded that these two cysteines are the active site residues that are involved in enzyme catalysis (21).

We confirmed that Cys-450 is part of a disulfide bond by limited trypsin digestion of carboxylase C598A/C700A which results in 30- and 60-kDa fragments (23). The two fragments of this fully active mutant were still joined by a disulfide bond (Fig. 8D). Therefore, since Cys-450 is the only cysteine remaining in the 60-kDa fragment, it must be involved in a disulfide bond. In order to further explore the role of cysteines 99 and 450 in carboxylase, we replaced them with either serine or alanine. In agreement with Pudota et al. (21), mutant enzymes C99S and C450A have significantly decreased carboxylase activity (Table II). We propose that the loss of activity is due to the involvement of these two cysteines as active site residues (21). Limited trypsin digestion of the carboxylase mutants, C450A or C99S, results in degradation of the protein (Fig. 8A), instead of the characteristic 30- and 60-kDa tryptic fragments observed in wild-type carboxylase (23). These results are consistent with the notion that the carboxylase’s structure is compromised by elimination of the disulfide bond.

Our result that cysteine residues 99 and 450 of carboxylase are joined by a disulfide bond differs from the result of Pudota et al. (21) in that they suggest that these two cysteines are the active site residues of carboxylase. They used radioactive [14C]NEM to modify the free cysteine residues of native carboxylase, quenched the reaction with dithiothreitol, denatured the NEM-modified carboxylase with urea, and modified the remaining cysteines with iodoacetamide. The [14C]NEM and iodoacetamide-modified carboxylase was then digested with trypsin and analyzed by LC-ESMS. Their results suggest that only cysteine residues 99 and 450 are modified by NEM (21). Since mutations of cysteines 99 and 450 dramatically decreased carboxylase activity, they concluded that these two cysteines are the active site residues that are involved in enzyme catalysis (21).

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cysteines 343 and 288. They reported that C343S resulted in an enzyme with 7—11 fold less activity than the wild type carboxylase and that C288S had normal activity. We found normal activity for C343S and 56% activity for C288S. In order to confirm that our mutation was correct, viral DNA from both mutants was extracted and the complete cDNA sequence reconfirmed. The difference in our results and theirs may be because Pudota et al. (21) used solubilized microsomes for their activity assay while we used purified enzyme preparations. Furthermore, they used a Western blot to determine the enzyme concentration, while we determined the active fraction of enzyme concentration, while we determined the active fraction of our enzyme preparation by titration with a fluorescent consensus propeptide (37).

In conclusion, we examined the status of all ten cysteine residues in human vitamin K-dependent γ-glutamyl carboxylase. Eight cysteines were found as free sulfhydryls. A disulfide bond joins only cysteines 99 and 450. Site-directed cysteine mutagenesis and limited trypsin digestion of the mutant carboxylase confirm the disulfide assignment. Since mutation of seven of the free cysteine residues has little effect on the enzyme activity and C288S has 56% of normal activity, it is possible that sulfhydryls are not involved in vitamin K dependent carboxylation.

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REFERENCES

1. Presnell, S. R., and Stafford, D. W. (2002) Thromb. Haemost. 87, 937—946
2. Price, P. A. (1988) Annu. Rev. Nutr. 8, 565—583
3. Brøstel, J. A., Ratcliffe, J. V., Roth, D. A., Jacobs, M. A., Furie, B. C., and Furie, B. (1996) Blood 88, 2585—2593
4. Carlisle, T. L., and Suttie, J. W. (1980) Biochemistry 19, 1161—1167
5. Vermeer, C. (1990) Biochem. J. 266, 625—636
6. Furie, B., Bouchard, A. B., and Furie, B. C. (1999) Blood 93, 1798—1808
7. Berkner, K. L. (2000) J. Nutr. 130, 1877—1880
8. Manfioletti, G., Brancolini, C., Avanzi, G., and Schneider, C. (1993) Mol. Cell. Biol. 13, 4976—4985
9. Kulman, J. D., Harris, J. E., Haldeman, B. A., and Davie, E. W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9058—9062
10. Kulman, J. D., Harris, J. E., Xie, L., and Davie, E. W. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 1370—1375
11. Tse, J., Wu, S. M., Jin, D., Niechitta, C. V., and Stafford, D. W. (2000) Blood 96, 973—978
12. Giles, N. M., Giles, G. I., Jacob, C. (2003) Biochem. Biophys. Res. Commun. 300, 1—4
13. Hunter, A., and Bardwell, J. C. (2003) Biochemistry 42, 1179—1185
14. Wedemeyer, W. J., Welker, E., Narayan, M., and Scheraga, H. A. (2000) Biochemistry 39, 4207—4216
15. Molinari, M., and Helenius, A. (1999) Nature 402, 90—93
16. Bartlett, G. J., Porter, C. T., Borkakoti, N., and Thornton, J. M. (2002) J. Mol. Biol. 324, 105—121
17. Morris, D. P., Soute, B. A., Veerum, C., and Stafford, D. W. (1993) J. Biol. Chem. 268, 8735—8742
18. Canfield, L. M. (1987) Biochem. Biophys. Res. Commun. 148, 184—191
19. Bouchard, B. A., Furie, B., and Furie, B. C. (1999) Biochemistry 38, 8517—8523
20. Canfield, L. M., Sinsky, T. A., and Suttie, J. W. (1980) Arch Biochem. Biophys. 202, 515—524
21. Pudota, B. N., Miyagi, M., Hallgren, R. W., West, K. A., Crab, J. W., Misono, K. S., and Berker, K. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13033—13038
22. Dowd, P., Hershline, R., Ham, S. W., and Naganathan, S. (1995) Science 269, 1684—1691
23. Wu, S. M., Mutucumaran, V. P., Geromans, S., and Stafford, D. W. (1997) J. Biol. Chem. 272, 11718—11722
24. Santoni, V., Molloy, M., and Rabilloud, T. (2000) Electrophoresis 21, 1054—1070
25. Wu, C. C., and Yates, J. R. (2003) Nat. Biotechnol. 21, 262—267
26. Stevens, T. J., and Arkin, I. T. (2000) Proteins 39, 417—420
27. Wallin, E., and von Heijne, G. (1996) Protein Sci. 5, 1029—1038
28. Garavito, R. M., and Ferguson-Miller, S. (2001) J. Biol. Chem. 276, 32403—32406
29. Wu, C. C., MacCoss, M. J., Howell, K. E., and Yates, J. R. (2003) Nat. Biotechnol. 21, 532—538
30. van Montfort, B. A., Canas, B., Duurkens, R., Godovac-Zimmermann, J., and Robillard, G. T. (2002) J. Mass Spectr. 37, 322—330
31. Sarkar, G., and Sommer, S. S. (1996) BioTechniques 8, 404—407
32. Datta, A. K. (1995) Nuclear Acids Res. 23, 4530—4531
33. Stearns, D. J., Kurosawa, S., Sims, P. J., Emon, N. L., and Emon, C. T. (1988) J. Biol. Chem. 263, 826—832
34. Stanley, T. B., Jin, D. Y., Lin, P. J., and Stafford, D. W. (1999) J. Biol. Chem. 274, 16940—16944
35. Pudota, B. N., Hommema, E. L., Hallgren, K. W., McNally, B. A., Lee, S., and Berker, K. L. (2001) J. Biol. Chem. 276, 46878—46886
36. Rudd, P. M., Joao, H. C., Coghill, E., Fiten, P., Saunders, M. R., Opdenakker, G., and Dwek, R. A. (1994) Biochemistry 33, 17—22
37. Presnell, S. R., Yip, S. Y., Lentz, B. R., Jin, D. Y., and Stafford, D. W. (2001) Biochemistry 40, 11723—11733
38. Clauser, K. R., Baker, P., and Burlingame, A. L. (1999) Anal. Chem. 71, 2871—2882
39. Patterson, S. D., and Katta, V. (1994) Anal. Chem. 66, 3727—3732
40. Jones, M. D., Patterson, S. D., and Lu, H. S. (1998) Anal. Chem. 70, 136—143
41. Crimmins, D. L., Saylor, M., Rush, J., and Thoma, R. S. (1995) Anal. Biochem. 236, 355—361
42. Zhou, J., Ens, W., Poppe-Schriemer, N., Standing, K. G., and Westmore, J. B. (1993) Int. J. Mass Spectr. Ion Processes 136, 115—122
43. Happersberger, H. P., Bantscheff, M., Babirz, S., and Glocker, M. O. (2000) Methods Mol. Biol. 146, 167—184
44. Schnaible, V., Wefing, S., Resemann, A., Suckau, D., Bucker, A., Wolf-Kumeth, S., and Hoffmann, D. (2002) Anal. Chem. 74, 4890—4888
45. Kim, J. S., and Kim, H. J. (2001) Rapid Commun. Mass Spectr. 15, 2296—2300
46. Nishida, A. S., Osuga, D. T., Lee, H. S., Ahmad, A. I., Whaitaker, J. R., and Feneley, R. E. (1977) J. Agric. Food Chem. 25, 245—251
47. Kaneko, R., and Kitabatake, N. (1999) J. Agric. Food Chem. 47, 4950—4955
Determination of Disulfide Bond Assignment of Human Vitamin K-dependent γ -Glutamyl Carboxylase by Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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