THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 46, Issue of November 14, pp. 45468–45475, 2003

Printed in U.S.A.

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

Received for publication, August 18, 2003
Published, JBC Papers in Press, September 8, 2003, DOI 10.1074/jbc.M309164200

Jian-Ke Tie, Vasantha P. Mutucumarana, David L. Straight, Kevin L. Carrick‡, R. Marshall Pope‡§, and Darrel W. Stafford¶

From the Departments of Biology and ‡Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599

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, 13033–13038 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 ^{14}C-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

* This work was supported by National Institutes of Health Grant HL48318 (to D. W. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Current address: Invitrogen Corp., Research and Development, 5781 Van Allen Way, Carlsbad, CA 92008.

¶ To whom correspondence should be addressed: Dept. of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3280. Tel.: 919-962-0597; Fax: 919-962-9266; E-mail: dws@email.unc.edu.

[1] The abbreviations used are: Gla, γ-carboxyglutamic acid; ER, endoplasmic reticulum; PRGP, proline-rich γ-carboxyglutamic acid proteins; TMG, transmembrane γ-carboxyglutamic acid proteins; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; PC, 1,2-dioleoyl-*sn*-glycero-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.

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 many 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 cysteines 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-hydroxysuccinnamic acid, and *N*-ethylmaleimide (NEM) were obtained from Sigma (St. Louis, MO). 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (PC) was from Avanti (Alabaster, AL). H-D-Phe-Pro-Arg-chloromethylketone and FLEEL were from Bachem (King of Prussia, PA). $NaH^{14}CO_3$ (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 K_1 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 Amersham Biosciences, and anti-HPC4 resin was kindly provided by Dr. Charles Esmon (Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK).

Site-directed Mutagenesis—Oligonucleotides and PCR primers used for site-directed mutagenesis were synthesized by Invitrogen Life Technologies (Frederick, MD). Site-directed cysteine mutagenesis of human carboxylase was conducted by 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 (EDQVDPRLIDGK) at the carboxyl terminus (33) was used as template DNA for the PCR. Mutations were screened by restriction digestion, verified by sequencing the entire cDNA of the carboxylase and subcloned to the expression vector pVL1392.

Expression and Purification of Human Carboxylase in Insect Cells—Wild-type or cysteine mutant carboxylase cDNA engineered in the expression vector was co-transfected with BacVector 3000 triple-cut virus DNA into Sf9 cells. The recombinant virus was isolated by plaque purification, amplified, and screened by carboxylase activity assay of the cell lysate. Expression of carboxylase was done by infection of $\sim 2 \times 10^6$/ml High Five cells with the recombinant virus at a mutiplicity of infection of ~1. Cells were collected after 48 h of infection, and the expressed carboxylase was purified by affinity chromatography using anti-HPC4 antibody-coupled Sepharose resin as described (34). Further concentration of the protein was accomplished by binding the affinity-purified enzyme to SP-Sepharose in 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1% PC, 0.3% CHAPS, and 15% glycerol, followed by step elution with 500 mM NaCl in the same buffer. The reducing reagent dithiothreitol was excluded from all purification steps.

Because our results on the activity of carboxylase with point mutations C343S and C288S differ from those of Pudota *et al.* (21, 35), we isolated the virus DNA from which these mutant proteins were prepared and the entire cDNA sequence was again confirmed.

Carboxylase Activity Assays—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 $(NH_4)_2SO_4$, 0.12% PC, 0.28% CHAPS, 4 μM proFIX, 5 μCi of $NaH^{14}CO_3$ (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 $NaH^{14}CO_3$ and vitamin K hydroquinone; the reaction mix was immediately transferred to a 20 °C water bath and incubated for 30 min. The reactions were terminated by the addition of 1 ml of 5% trichloroacetic acid, and the amount of $^{14}CO_2$ incorporated into the small substrate FLEEL was determined, as previously described (34).

Deglycosylation and NEM Modification of the Carboxylase—Glycoproteins often yield poor peptide maps for mass analysis because the oligosaccharides can effectively shield proteolytic cleavage sites (36). Therefore, freshly purified carboxylase was deglycosylated before being subjected to protease digestion by adding 100 μl of Buffer G7 (50 mM sodium phosphate, pH 7.5 at 25 °C) and 10 μl of PNGase F (500 units/μl) to a 900-μl carboxylase sample. Three hours at 37 °C was sufficient for deglycosylation of denatured carboxylase; however, 16 h at 18 °C was required for deglycosylation of native carboxylase. The efficiency of deglycosylation was evaluated by SDS-PAGE. NEM modification of carboxylase was performed at room temperature, as described by Pudota *et al.* (21), except, instead of quenching the reaction by dithiothreitol, it was loaded directly onto non-reducing SDS-PAGE and the NEM used in our experiments was not radioactively labeled.

In-gel Protease Digestion—Carboxylase samples were loaded onto 10% SDS-PAGE gels in the absence of reducing reagent. After separation, the gel was fixed with 25% isopropyl alcohol/10% acetic acid for 20 min and stained with 0.01% Coomassie Brilliant Blue R250 in 10% acetic acid for 1 h. It was destained with 10% acetic acid, the protein band excised, and the gel pieces treated as described (30). The gel pieces were completely destained with 50 mM NH_4HCO_3 in 40% ethanol, and then washed with 1000 μl of 25 mM NH_4HCO_3 three times for 15 min and cut into pieces of <1 mm^3. Subsequently, the gel pieces were dehydrated with 1000 μl of acetonitrile three times for 10 min and completely dried with a SpeedVac.

Protease digestion was started by the addition of sufficient 100 ng/μl sequencing grade-modified trypsin or chymotrypsin in 25 mM NH_4HCO_3 buffer to immerse the dried gel pieces. After re-hydration, the gel pieces were covered with an overlay of 20 μl of 25 mM NH_4HCO_3 buffer so that they remained immersed throughout the digestion. The protein was digested overnight by trypsin at 30 °C without agitation. Digestion reaction supernatants were directly pooled for MALDI-TOF mass spectrum. After collecting the supernatant, the digested gel pieces were extracted three times by sonication for 5 min in 60% acetonitrile, 0.1% trifluoroacetic acid, and 0.1% *n*-octyl-β-D-glucopyranoside. These extracts were combined and dried in a SpeedVac. The dried sample was dissolved in 5 μl of 50% acetonitrile, 0.1% trifluoroacetic acid, sonicated for 2 min, and used for recording the MALDI-TOF mass spectrum. Chymotrypsin digestion was as above, with the exception that digestion was performed for 5 h and the reaction was stopped by adding trifluoroacetic acid to a final concentration of 5%. Reaction supernatant and extracts were combined and concentrated to ~20 μl by SpeedVac. The pH was adjusted by NH_4HCO_3 if needed. Reduced samples were prepared by adding freshly prepared TCEP to a final concentration of 2 mM to the non-reduced samples.

MALDI-TOF Mass Spectrometry—0.3 μl of the above sample and 0.3 μl of 10 mg/ml 1-cyano-4-hydroxysuccinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid (v/v) were deposited on the target plate and air-dried. MALDI mass spectra were recorded with an ABI 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA) MALDI-TOF/TOF mass spectrometer. A frequency-tripled Nd:YAG laser ionized samples at a pulse frequency of 200 Hz with its power adjusted between 15 and 30 μJ, depending on the sample. Laboratory air was used as the collision gas and the collision cell vacuum pressure was $7–9 \times 10^{-7}$ Torr. MALDI-TOF data from tryptic digests were calibrated with autoproteolytic peaks (internal standards) and mass errors were less than 20 ppm. The mass range for TOF MS scan functions was set to *m/z* 500 to 4000. TOF MS/MS scan functions were calibrated externally against the fragments of either angiotensin I or adrenocorticotropic hormone fragment 18–39 depending upon the precursor mass. The mass accuracy of CID data was typically better than 50 ppm.

Limited Trypsinization of Carboxylase—Limited trypsin digestions of carboxylase in the presence of propeptide were performed as previously described (23). A pilot reaction was run for each batch of trypsin

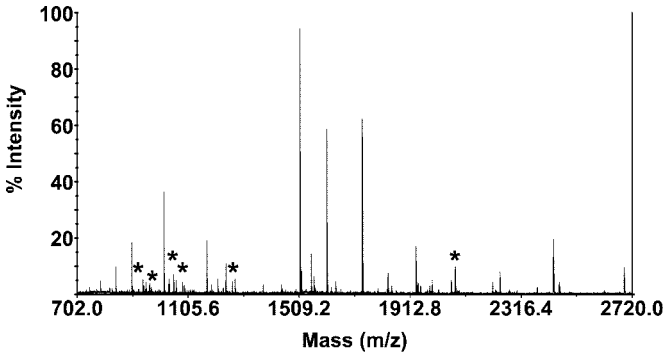

FIG. 1. **MALDI-TOF mass spectrum of the supernatant from in-gel trypsin digestion of non-reduced carboxylase.** *Asterisks* indicate the position of cysteine-containing peptides.

TABLE I
Assignment of fragment ions obtained from MALDI-TOF MS of the in-gel trypsin digestion of human vitamin K-dependent γ-glutamyl carboxylase

Residues	Measured mass	Expected mass
	m/z	*m/z*
407-412	735.3536	735.3902
84-90	747.3871	747.4001
666-671	787.4305	787.4314
674-680	900.4328	900.4328
−1-9	906.4529	906.4467
446-453[a]	941.4496	941.4515
327-334	952.6205	952.6195
319-326[a]	965.4939	965.4879
319-326[a,b]	1036.5276	1036.5250
92-100[a]	1053.5046	1053.5039
673-680	1056.5461	1056.5339
235-242	1061.5537	1061.5573
20-29	1085.6000	1085.5955
92-100[a,b]	1124.5502	1124.5410
437-445[c]	1143.5422	1143.5621
579-588	1173.5836	1173.5727
335-346[a]	1249.6167	1249.6251
648-661	1511.8230	1511.8222
486-498	1516.7998	1516.7912
192-204	1552.8467	1552.8389
37-49	1609.8048	1609.8015
420-435	1738.8784	1738.8764
69-83[c]	1824.9742	1824.9722
499-513	1828.9561	1828.9420
219-234	1933.8737	1933.8642
51-68	1983.1403	1983.1391
218-234	2061.9592	2061.9592
689-704[a,b]	2076.0830	2076.1251
458-476[d]	2373.1978	2372.1563
625-647[d]	2433.2903	2432.2561
623-647[d]	2690.4270	2689.3936

[a] Cysteine-containing peptides.
[b] Acrylamide modified cysteine residue containing peptide.
[c] Peptides recovered from the gel extract.
[d] Glycosylated peptides.

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-D-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"

1 **MAVSAGSAR**T SPSSDKVQK**D KAELISGPR**Q DSRIGK**LLGF EWTDLSSWRR**

51 **LVTLLNRPTD PASLAVFR*FL FGFLMVLDIP* *Q*ERGLSSLDR KYLDGLDVCR**

101 FPLLDALRPL PLDWMYLVYT IMFLGALGMM LGLCYRISCV LFLLPYWYVF

151 LLDKTSWNNH SYLYGLLAFQ LTFMDANHYW SVDGLLNAHR R**NAHVPLWNY**

201 **AVLR**GQIFIV YFIAGVK**KLD ADWVEGYSME YLSRHWLFSP FK**LLLSEELT

251 SLLVVHWGGL LLDLSAGFLL FFDVSRSIGL FFVSYFHCMN SQLFSIGMFS

301 YVMLASSPLF CSPEWPRK**LV SYCPQRLQQL LPLKAAPQPS VSCVYK**RSRG

351 KSGQKPGLRH QLGAAFTLLY LLEQLFLPYS HFLTQGYNNW TNGLYGYSWD

401 MMVHSR**SHQH VK**ITYRDGR**T GELGYLNPGV FTQSRR*WKDH ADMLK*QYATC**

451 **LSR**LLPK**YNV TEPQIYFDIW VSINDR**FQQR IFDPR**VDIVQ AAWSPFQRTS**

501 **WVQPLLMDLS PWR**AKLQEIK SSLDNHTEVV FIADFPGLHL ENFVSEDLGN

551 TSIQLLQGEV TVELVAEQKN QTLREGEK**MQ LPAGEYHK**VY TTSPSPSCYM

601 YVYVNTTELA LEQDLAYLQE LK**EKVENGSE TGPLPPELQP LLEGEVKGGP**

651 **EPTPLVQTFL R**RQQR**LQEIE RRRNTPFHER** FFRFLLRK**LY VFRRSFLMTC**

701 **ISLR**NLILGR PSLEQLAQEV TYANLRPFEA VGELNPSNTD SSHSNPPESN

751 PDPVHSEF

FIG. 2. **Amino acid sequence of human γ-glutamyl carboxylase showing tryptic and chymotryptic peptide fragments detected by MALDI-TOF MS.** *Boldface* residues, recovered from the trypsin digestion supernatant. Residues in *boldface italic* were recovered from an additional extract of trypsin-digested gel plugs. *Underlined* residues were recovered from chymotrypsin digestion. *Boxed regions* represent transmembrane segments according to our recent carboxylase topology study (11). The sequence coverage for tryptic digestion is 41.9%. Chymotryptic sequence coverage is 30.5%. The combined sequence coverage is 62.3%.

Version 4.0.4 (available at prospector.ucsf.edu), was used to identify the mass peaks in the "NCBInr.5.28.2003" 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 ^{18}O-labeled water (data not shown).

To increase the sequence coverage, limited chymotrypsin digestion of carboxylase was performed. Chymotrypsin 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.

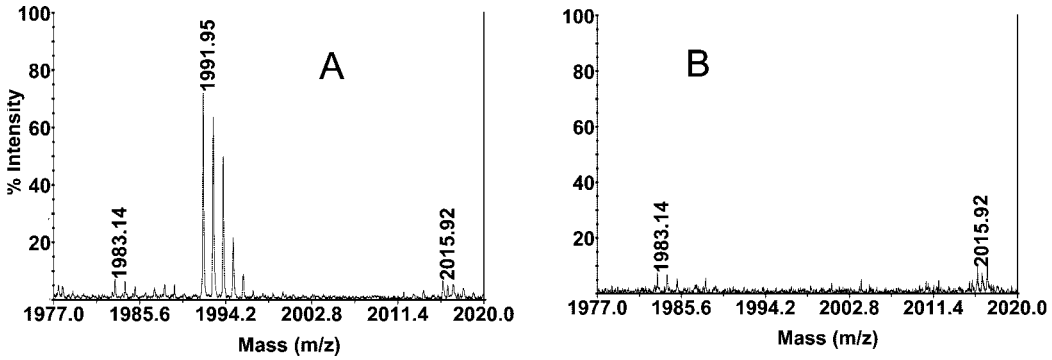

FIG. 3. **MALDI-TOF mass spectra of the supernatant of the in-gel trypsin digestion of carboxylase.** *Panel A*, detail of the spectrum shown in Fig. 1 from *m/z* 1977.0 to 2020.0 (Non-reduced sample). *Panel B*, spectrum of a TCEP-reduced sample in the same *m/z* range shown in *panel A*.

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. 3*A*) 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. 3*B*). 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. 4*B*). 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

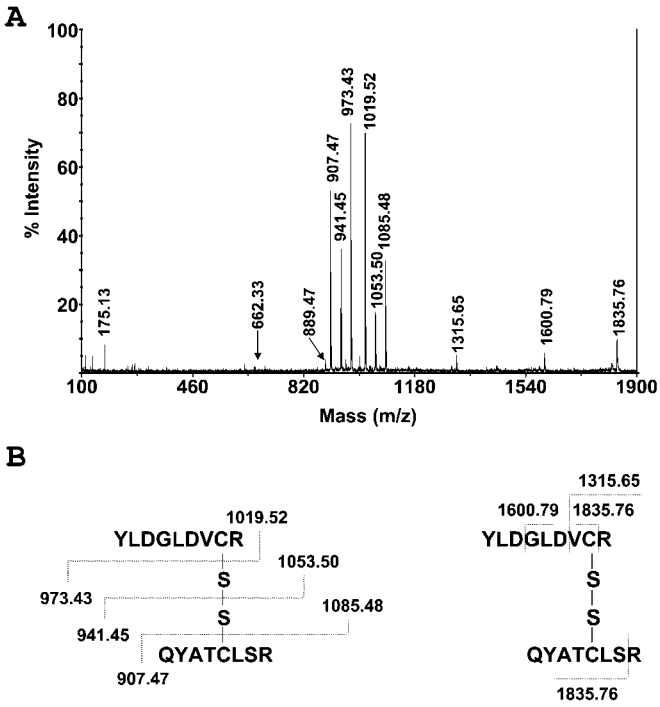

FIG. 4. **MS/MS spectrum of the disulfide-linked precursor ion contaning Cys-99 and Cys-450.** *Panel A*, fragmentation spectrum following CID of the MS peak at *m/z* 1991.9. *Panel B*, the diagrammatical interpretation of the fragmentation of disulfide-linked peptide 92–100 and 446–453.

(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 chymotrypin-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

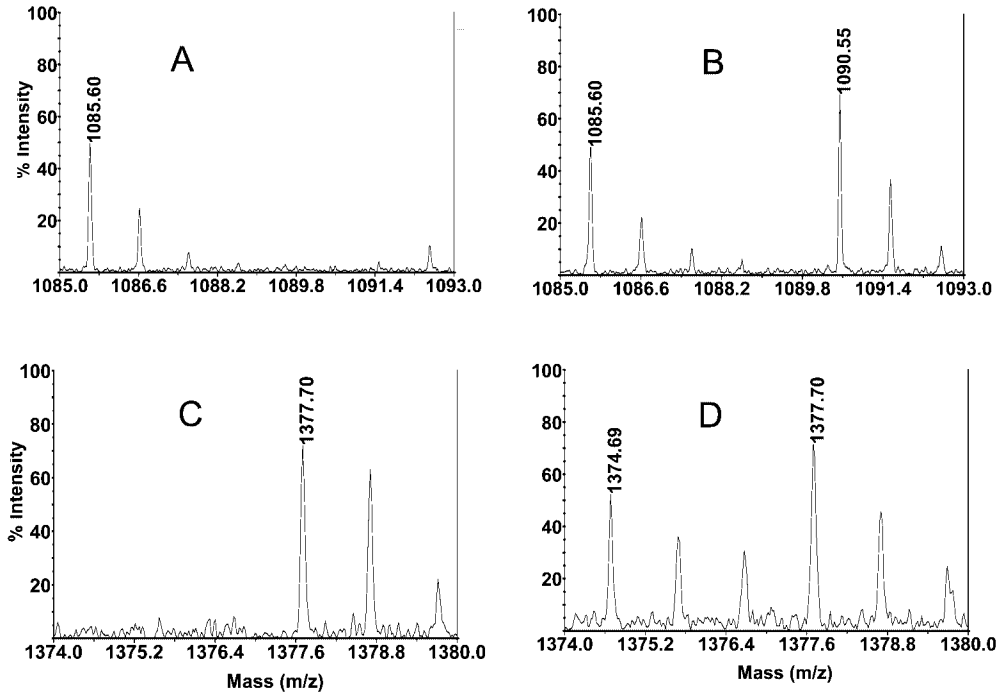

FIG. 5. **Comparison of the MALDI-TOF mass spectra of the trypsin digestion of carboxylase and NEM-modified carboxylase (NEM modification of Cys-323 and Cys-343).** *Panel A*, unmodified carboxylase spectrum from *m/z* 1085.0 to 1093.0. *Panel B*, NEM-modified carboxylase spectrum from *m/z* 1085.0 to 1093.0. *Panel C*, unmodified carboxylase spectrum from *m/z* 1374.0 to 1380.0. *Panel D*, NEM-modified carboxylase spectrum from *m/z* 1374.0 to 1380.0. Peptide ions at *m/z* 1090.55 (*panel B*) and 1374.69 (*panel D*) correspond to the NEM-labeled peptides containing cysteines 323 and 343, respectively.

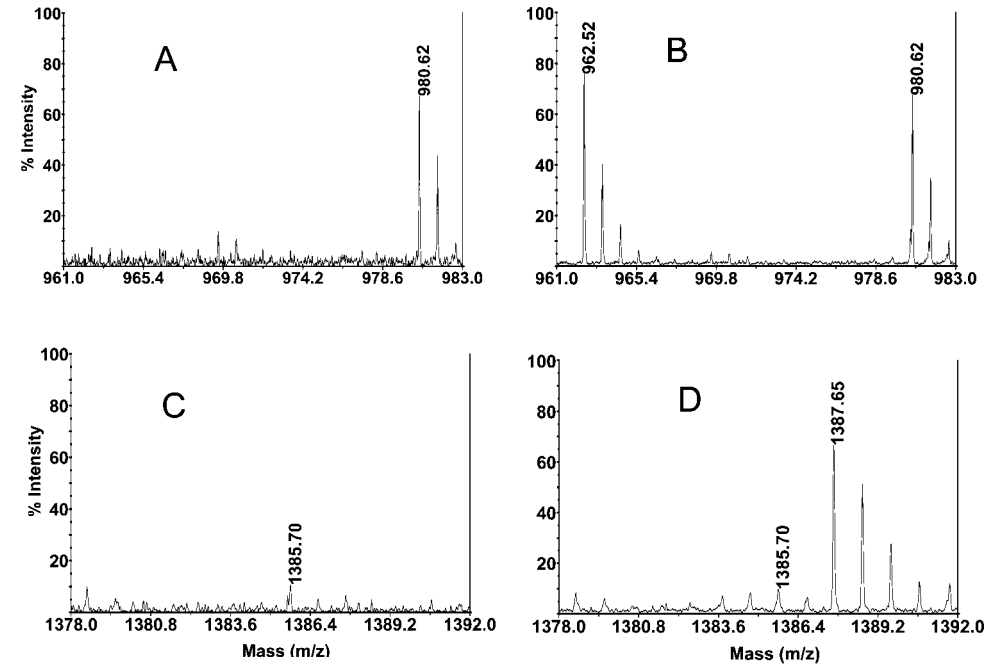

FIG. 6. **Comparison of the MALDI-TOF mass spectra of the chymotrypsin-digested carboxylase and NEM-modified carboxylase (NEM modification of Cys-139 and Cys-311).** *Panel A*, unmodified carboxylase spectrum in the *m/z* range from 961.0 to 983.0. *Panel B*, NEM-modified carboxylase spectrum from *m/z* 961.0 to 983.0. *Panel C*, unmodified carboxylase spectrum from *m/z* 1378.0 to 1392.0. *Panel D*, NEM-modified carboxylase spectrum from m/z 1378.0 to 1392.0. Peptide ions appear at *m/z* 962.52 (*panel B*) and 1387.65 (*panel D*), corresponding to the NEM-labeled peptides containing cysteines 139 and 311, respectively.

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. 8*A*, *lane 2*) is more degraded than the wild-type enzyme (Fig. 8*A*, *lane 1*) and instead of the expected 30- and 60-kDa

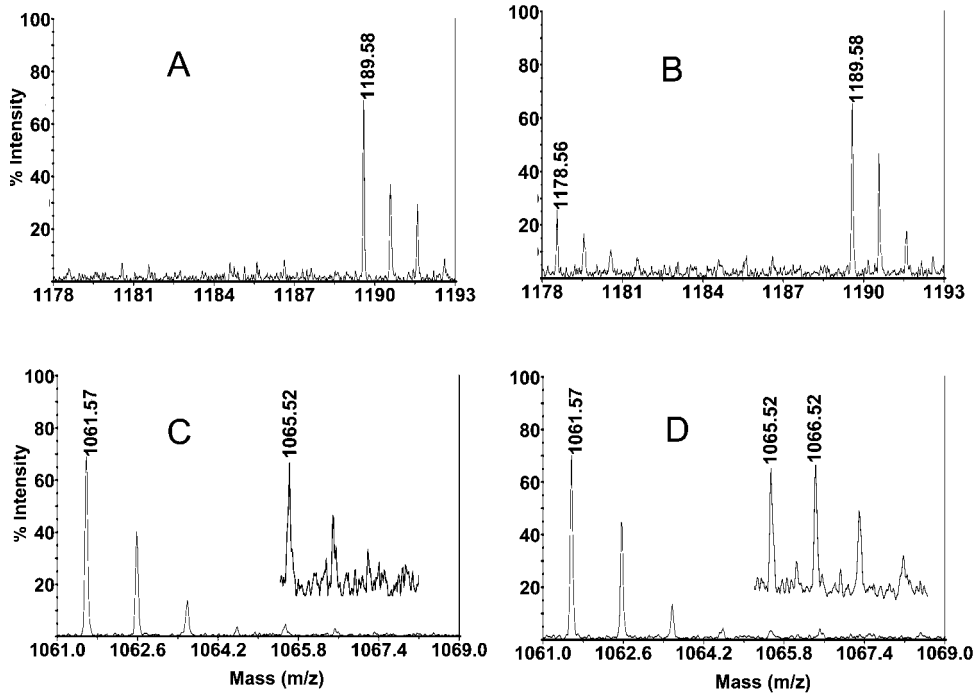

FIG. 7. **Comparison of the MALDI-TOF mass spectra of the trypsin digestion of carboxylase and NEM-modified carboxylase (NEM modification of Cys-99 and Cys-450).** *Panel A*, unmodified carboxylase spectrum from *m/z* 1178 to 1193. *Panel B*, NEM-modified carboxylase spectrum from *m/z* 1178 to 1193. *Panel C*, unmodified carboxylase spectrum from *m/z* 1061.0 to 1069.0. *Panel D*, NEM-modified 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.

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
	$^{14}CO_2$ pmol/h/pmol enzyme	
WT	1192 ± 17	100
C99S	6.7 ± 0.63	0.56
C134W	1186 ± 11	99.5
C139S	1061 ± 13	89.0
C288S	667 ± 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

fragments, limited trypsin digestion results in further degradation (Fig. 8*A*, *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. 8*B*, *lane 5*) when analyzed by SDS-PAGE. When the sample is reduced, however, the 30- and 60-kDa tryptic fragments appear (Fig. 8*B*, *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 spectrum of the non-reduced sample (*m/z* at 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 thiocysteine 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. Although the data is not shown, fragmentation of both the 92–100 and 446–453 component peptides in the reduced form of the trypsin-digested sample confirms the assignment and provides further proof for the disulfide linkage be-

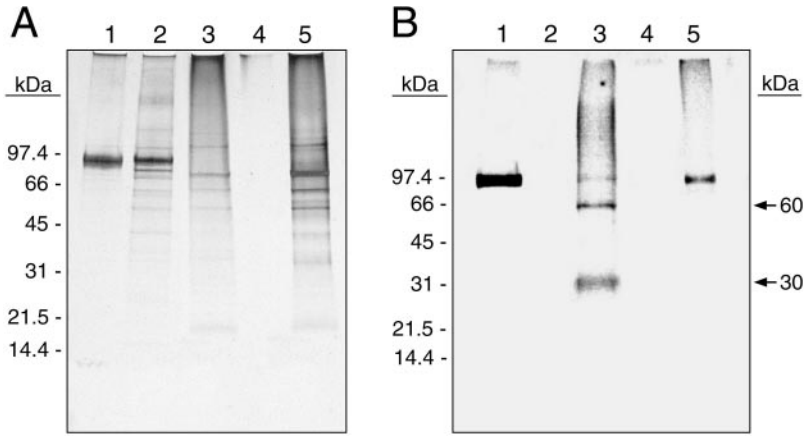

FIG. 8. **Limited trypsin digestion of cysteine mutant carboxylases C450A and C598A/C700A.** *Panel A*, *lane 1*, intact wild-type carboxylase; *lane 2*, intact C450A; *lane 3*, trypsinized C450A, reduced; and *lane 5*, trypsinized C450A, non-reduced. *Panel B*, *lane 1*, intact C598A/C700A; *lane 3*, trypsinized C598A/C700A, reduced, and *lane 5*, trypsinized C598A/C700A, non-reduced.

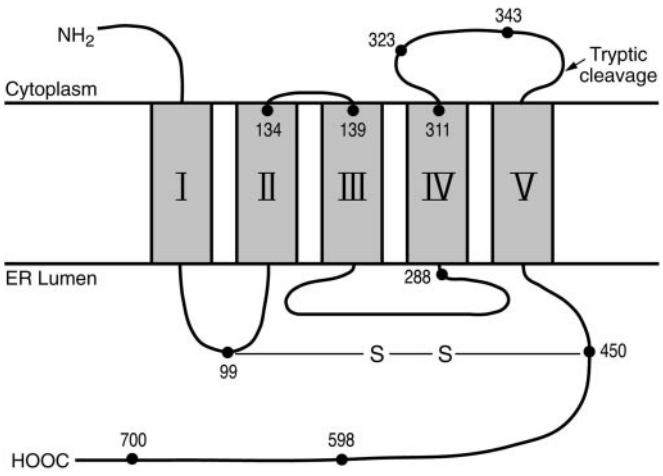

FIG. 9. **Membrane topology of vitamin K-dependent γ-glutamyl carboxylase based on our recent study (11).** The positions of all ten cysteine residues are marked. Disulfide linkage between cysteine residues 99 and 450 is based upon the results of the current study. Limited tryptic cleavage occurs at amino acid residue 349 and 351.

tween cysteine residues 99 and 450.

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. 8*B*). 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 structural changes caused by removal of the disulfide bond, rather than being 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. 8*A*), 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 [^{14}C]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 [^{14}C]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).

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 cysteine residues 99 and 450 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 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 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 assays while we used purified enzyme preparations. Furthermore, they used a Western blot to determine the 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.

Acknowledgments—We thank Dayun Jin and Chun Yun Chang for their excellent technical assistance.

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. Bristol, 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, B. A., 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. Tie, J., Wu, S. M., Jin, D., Nicchitta, C. V., and Stafford, D. W. (2000) *Blood* **96,** 973–978
12. Giles, N. M., Giles, G. I., and Jacob, C. (2003) *Biochem. Biophys. Res. Commun.* **300,** 1–4
13. Hiniker, 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., Vermeer, 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,** 9517–9523
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, K. W., West, K. A., Crabb, J. W., Misono, K. S., and Berkner, 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., Mutucumarana, V. P., Geromanos, 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. (1998) *Protein Sci.* **7,** 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. (1990) *BioTechniques* **8,** 404–407
32. Datta, A. K. (1995) *Nucleic Acids Res.* **23,** 4530–4531
33. Stearns, D. J., Kurosawa, S., Sims, P. J., Esmon, N. L., and Esmon, 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 Berkner, 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., Tripathy, A., 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.* **226,** 355–361
42. Zhou, J., Ens, W., Poppe-Schriemer, N., Standing, K. G., and Westmore, J. B. (1993) *Int. J. Mass Spectr. Ion Processes* **126,** 115–122
43. Happersberger, H. P., Bantscheff, M., Barbirz, S., and Glocker, M. O. (2000) *Methods Mol. Biol.* **146,** 167–184
44. Schnaible, V., Wefing, S., Resemann, A., Suckau, D., Bucker, A., Wolf-Kummeth, S., and Hoffmann, D. (2002) *Anal. Chem.* **74,** 4980–4988
45. Kim, J. S., and Kim, H. J. (2001) *Rapid Commun. Mass Spectr.* **15,** 2296–2300
46. Nashef, A. S., Osuga, D. T., Lee, H. S., Ahmed, A. I., Whitaker, J. R., and Feeney, R. E. (1977) *J. Agric. Food Chem.* **25,** 245–251
47. Kaneko, R., and Kitabatake, N. (1999) *J. Agric. Food Chem.* **47,** 4950–4955