Chemical Modification of Cysteine Residues Is a Misleading Indicator of Their Status as Active Site Residues in the Vitamin K-dependent γ-Glutamyl Carboxylation Reaction*

Received for publication, August 4, 2004, and in revised form, October 13, 2004 Published, JBC Papers in Press, October 18, 2004, DOI 10.1074/jbc.M408945200

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The enzymatic activity of the vitamin K-dependent proteins requires the post-translational conversion of specific glutamic acids to γ-carboxy-glutamic acid by the integral membrane enzyme, γ-glutamyl carboxylase. Whether or not cysteine residues are important for carboxylase activity has been the subject of a number of studies. In the present study we used carboxylase with point mutations at cysteines, chemical modification, and mass spectrometry to examine this question. Mutation of any of the free cysteine residues to alanine or serine had little effect on carboxylase activity, although C343A mutant carboxylase had only 38% activity compared with that of wild type. In contrast, treatment with either thiol-reactive reagent 4-acetamido-4'-maleimidyl-stilbene-2,2'-disulfonic acid, disodium salt, or sodium tetrathionate, caused complete loss of activity. We identified the residues modified, using matrix-assisted laser desorption/ionization time of flight mass spectrometry, as Cys323 and Cys343. According to our results, these residues are on the cytoplasmic side of the microsomal membrane, whereas catalytic residues are expected to be on the luminal side of the membrane. Carboxylase was partially protected from chemical modification by factor IXs propeptide. Although all mutant carboxylases bound propeptide with normal affinity, chemical modification caused a 100-fold decrease in carboxylase affinity for the consensus propeptide. We conclude that cysteine residues are not directly involved in carboxylase catalysis, but chemical modification of Cys323 and Cys343 may disrupt the three-dimensional structure, resulting in inactivation.

Vitamin K-dependent carboxylation is required for the biological activity of vitamin K-dependent proteins. Carboxylation is a post-translational conversion of glutamyl residues in the precursor proteins to γ-carboxyglutamic acid (gla) residues in the mature protein (1, 2). These gla residues allow the vitamin K-dependent proteins to bind calcium necessary for their biological functions in blood coagulation, bone homeostasis, and other areas. Vitamin K-dependent carboxylation is catalyzed by an integral membrane glycoprotein, γ-glutamyl carboxylase. During the process of carboxylation, the γ-hydrogen of the glutamic acid is abstracted, followed by the addition of CO₂ (1). Simultaneously, carboxylase converts vitamin K hydroquinone (VKH₂) to vitamin K 2,3-epoxide (vitamin K epoxidation) (3, 4).

Numerous publications have suggested that free cysteine residues are important for vitamin K-dependent carboxylation and epoxidation. For example, the addition of DTT to carboxylase-containing microsomes stimulates carboxylation of glutamyl residues (5–8). Moreover, modification of carboxylase by sulfhydryl-reactive reagents inactivates the enzyme (3, 8–11). Canfield (9) reported that a thiol group is essential for the binding of VKH₂ to the active site of carboxylase. Bouchard et al. (10) showed that NEM, a sulfhydryl-reactive reagent, reacts with two to three free cysteines in bovine carboxylase, resulting in the inactivation of the enzyme. Further support for this hypothesis was provided by Pudota et al. (11), who used electrospray mass spectrometry to identify the cysteine residues modified by NEM. They concluded that Cys³⁹⁹ and Cys⁴⁵⁰ are the two active site cysteines required for both vitamin K-dependent carboxylation and epoxidation.

Based on the observations that carboxylase is inactivated by sulfhydryl-reactive reagents, Dowd et al. (12) used a nonenzymatic chemical model to develop a “base strength amplification mechanism” for vitamin K-dependent carboxylation. Those investigators proposed that two free cysteines are involved in the active site of the carboxylase.

We recently found that Cys³⁹⁹ and Cys⁴⁵⁰ are actually joined in a disulfide bond (13). In addition, we found that mutation of each of the other eight cysteine residues in carboxylase did not inactivate the enzyme, although C288S had only 56% activity relative to that of wild type. Although we confirmed that NEM modification of carboxylase causes inactivation, mutation of the modified residues Cys³⁹⁹, Cys³¹¹, Cys³²³, and Cys³⁴³ did not inactivate the carboxylase (13). These results raise the question of whether sulfhydryl groups are really important for catalysis, and if so by what mechanism? In our first study we used DTT, the peptide Phe-Leu-Glu-Glu-Leu; proFX, 19 amino acid residues of factor IX propeptide (18 to 1); proFXgla, propeptide with the gla domain of factor IX (18 to 46 with the mutations of R-4Q and R-1S); proCon, consensus propeptide (AVFLKSVKQNLQRRRR); MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MS, mass spectrometry.
changed cysteine to serine in most cases, so here we made cysteine to alanine changes. In addition to mutations, we employed thiol-reactive reagents with different charge/polarity characteristics to identify which cysteine residues in the carboxylase affect the enzyme activity by chemical modification. We used MALDI-TOF MS to identify the chemically modified cysteine residues. Our results suggest that modification of cysteines probably causes structural or steric effects on carboxylase rather than modifying residues directly involved in the carboxylation reaction. While this paper was under review, Rishavy et al. (14) reached a similar conclusion: that cysteine is not an active site residue of vitamin K-dependent carboxylase.

**EXPERIMENTAL PROCEDURES**

**Materials—**All of the chemicals were reagent grade. AMS and DABMI were obtained from Molecular Probes (Eugene, OR). NEM, phenylmethylsulfonyl fluoride, α-cyano-4-hydroxysuccinimide acid, Na$_2$S$_4$O$_6$, NaBH$_4$, and CHAPS were obtained from Sigma. Pentapeptide FLEEL and protease inhibitors, H-ν-Phe-Arg-chloromethylketone and H-ν-Arg-Pro-Arg chloromethylketone were from Bachem (King of Prussia, PA). 1,2-Dioleyl-sn-glycerol-3-phosphocholine was from Avanti (Alabaster, AL). The protease BstUI was from New England Biolabs. Phosphate-buffered saline, 2.4% w/v bovine serum albumin, and 0.1% sodium azide were prepared as described previously (15). Vitamin K$_1$ (10 mg/ml), which contains 70 mg/ml polysulfated fatty acid derivative, 37.5 mg/ml dextrose hydros, and 9 mg/ml benzylic alcohol was from Abbott Laboratories (Chicago, IL). Aprotinin, peptatin A, trypsin, and proteinase K were purchased from Roche Applied Science. Polyvinylidene fluoride transfer membrane was from Millipore Co. (Bedford, MA). NaH$_4$CH$_3$(COOH)$_3$ (specific activity, 54 Ci/ml) was from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Restriction enzymes and peptide N-glycosidase F were from New England Biolabs (Beverly, MA). Anti-HPC4 resin was kindly provided by Dr. Charles Esmon (Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK).

**Reduction of Vitamin K$_1$ to VKH$_2$—**Three volumes of buffer K (500 mM NaCl, 20 mM Tris-HCl, pH 8.5) containing 200 mM DTT were added to one volume of vitamin K$_1$ solution (10 mg/ml) and incubated in the dark at 37 °C for at least 24 h to ensure complete reduction. The concentration of this reduced VKH$_2$ stock solution was 5.55 mM. For experiments where DTT was avoided, vitamin K$_1$ was freshly reduced by NaBH$_4$ as described previously (16). The reduced VKH$_2$ buffer K, and NaBH$_4$ was added to the solution to obtain a final concentration of 0.1 M. The reduction reaction was incubated in the dark at room temperature for 20 min. Excess NaBH$_4$ was removed by adjusting the reaction to pH 4.0 with 1 M HCl and incubating at room temperature for 5 min. Then the pH of the solution was adjusted to pH 8.5 with 1 M Tris, and buffer K was added to adjust the concentration of VKH$_2$ to 5.55 mM.

**Carboxylase Activity Assays—**The carboxylase activity was determined by the incorporation of $^{34}$CO$_2$ into the pentapeptide substrate FLEEL. The assay was performed in 25 mM MOPS (pH 6.8), 500 mM NaCl, 0.8 mM (NH$_4$)$_2$SO$_4$, 0.12% 1,2-dioleyl-sn-glycerol-3-phosphocholine, 0.28% CHAPS (buffer A), with 4 mM proFIX, and 1.25 mM FLEEL. The reaction was started by the addition of 10 μl of an ice-cold mix of NaH$_4$CH$_3$(COOH)$_3$ (final concentration, 40 μM/ml) and VKH$_2$ (final concentration, 222 mM) to bring the volume to 125 μl. 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 $^{34}$CO$_2$ incorporation was determined as previously described (17).

**Sulfhydryl-reactive Reagent Modification of Carboxylase—**Purified carboxylase (50 nm) (16) was modified by the sulfhydryl-reactive reagents in buffer A. The reaction was initiated by the addition of the sulfhydryl-reactive reagent, and samples were incubated on ice for 20 min in the dark. The reaction was stopped by the addition of DTT to a final concentration of 50 mM. Carboxylase activity was determined by the incorporation of $^{34}$CO$_2$ into the pentapeptide substrate FLEEL (16). Na$_2$S$_4$O$_6$ modification was reversed by DTT. Therefore, when we used Na$_2$S$_4$O$_6$ as the thiol-reactive reagent, we stopped the reaction by diluting the sample 10-fold with buffer A. The enzyme activity was determined immediately as described above except we used NaBH$_4$-reduced VKH$_2$.

For sequential modification of carboxylase by Na$_2$S$_4$O$_6$ and AMS, carboxylase was first incubated with Na$_2$S$_4$O$_6$ (5 mM, on ice for 10 min). A portion of the sample was incubated with 1 mM AMS for another 10 min. When we wanted to reverse the Na$_2$S$_4$O$_6$ modification, we stopped the reaction by the addition of DTT (final concentration, 50 mM); otherwise we stopped the reaction with dilution as described above. The residual carboxylase activity was measured as above.

**Limited Trypsinization of Carboxylase—**Limited trypsin digestion of carboxylase was performed as previously described (17). A pilot reaction was run for each batch of trypsin and carboxylase to determine the optimal conditions for limited cleavage. Cleavage was accomplished on ice in the presence of 5 μM proFIX. We omitted proFIX in the trypsin digestion samples used for pepptide binding and protection experiments. Trypsin cleavage was stopped by the addition of H-ν-Phe-Pro-Arg-chloromethylketone and aprotinin to a final concentration of 1.6 μM. We analyzed the extent of trypsin cleavage of carboxylase on gradient (4–20%, reducing conditions) SDS-PAGE gels followed by silver staining. This limited trypsin-digested carboxylase is still enzymatically active.

**Protection of Carboxylase from Inactivation by Na$_2$S$_4$O$_6$—**We investigated whether the following molecules would protect carboxylase from inactivation by Na$_2$S$_4$O$_6$: 10 mM FLEEL, 8 μM proFIX, 8 μM proFIXglA, 222 μM VKH$_2$, and various combinations of these reagents. Carboxylase (50 nm) was incubated with the reagent(s) on ice for 5 min in buffer A. The samples were divided in equal volumes, and Na$_2$S$_4$O$_6$ (500 μM) was added to each sample. Then another 5 min incubation was added to the other. The mixtures were incubated on ice for 20 min, and then carboxylase activity was determined using NaBH$_4$-reduced VKH$_2$.

We measured the time course of inactivation of carboxylase and trypsin-digested carboxylase (50 nm) in the presence (8 μM proFIX) or absence of propeptide. We incubated the enzyme with or without propeptide on ice for 5 min. Na$_2$S$_4$O$_6$ was added, portions were removed, and the reaction was stopped at various times. The residual activity was determined as described above.

**Fluorescence Titration of Intact and Trypsin-digested Carboxylase—**Fluorescence anisotropy titration of intact and trypsin-digested carboxylase with 5(6)-carboxyfluorescein-labeled proFIX was performed on an OLIS-modified T-format SLM spectrophotometer (On-Line Instruments, Bogaart, GA) essentially as described previously (18). The excitation wavelength was set at 490 nm, and the slits were set at the 1-nm band pass. Two photon counters were used to detect the emissions at horizontal and vertical directions to increase the sensitivity. All of the measurements were performed in a 400-μl fluorimetric quartz cuvette at 4.5 °C in a final sample volume of 300 μl of buffer containing 100 mM MOPS, pH 7.5, 180 mM NaCl, 3.5% glycerol, 0.1% 1,2-dioleyl-sn-glycer-3-phosphocholine, 0.28% CHAPS, 0.4% bovine serum albumin (5 mg/ml), and 100 mM proFIX. After measuring fluorescence anisotropy, we converted the values to the fraction of enzyme bound. The $K_v$ values of proFIX for carboxylase were estimated as described previously (18). For titration of Na$_2$S$_4$O$_6$-inactivated intact and trypsin-digested carboxylase, 5(6)-carboxyfluorescein-labeled proCon was used as ligand.

**Identification of AMS-labeled Cysteine Residue of Carboxylase—**Carboxylase or Na$_2$S$_4$O$_6$-inactivated carboxylase were incubated with 1 mM AMS on ice for 20 min. The reactions were stopped by DTT. AMS-labeled and nonlabeled carboxylase samples were subjected to SDS-PAGE. The protein bands were fixed by soaking the gel in 25% isopropanol with 10% acetic acid for 30 min, stained with 0.01% Coomassie R-250 in 10% acetic acid for 1 h, and destained with 10% acetic acid. The carboxylase bands were excised, and in-gel dglycosylation and trypsin digestion were performed as described previously (13, 19). Trypsin digestion reaction supernatants were directly pooled for MALDI-TOF mass spectrometry (13). The digested sample (0.3 μl) and 0.3 μl of 10 mg/ml 1-cyano-4-hydroxysuccinimide 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 AB 4700 MALDI Analyst (Applied Biosystems, Foster City, CA). MALDI-TOF/MOD mass spectrometer as described (13). MALDI-TOF data from trypptic digests were calibrated with autoproteolytic peaks (internal standards), and mass errors were less than 20 ppm. TOF MS/MS scan functions were calibrated externally against the fragments of either angiotensin I or adrenocorticotropic hormone fragment 18–39 depending upon the specificity of the applied collision-induced dissociation data was typically better than 50 ppm.

**Identification of the Last Transmembrane Domain of Carboxylase—**Freshly prepared carboxylase-containing microsomes from High Five cells were digested by protease K as described previously (20). The reaction was stopped by the addition of phenylmethylsulfonyl fluoride (final concentration, 3 mM). The protease should degrade all of the peptide loops of carboxylase on the outside of the micosomal membra
brane, leaving the sequences inside the membranes intact. The microsomes were washed 3 times with the digestion buffer containing 3 mM phenylmethylsulfonyl fluoride. According to our previously published membrane topology (20), the C-terminal fragment with the last transmembrane domain of carboxylase should contain the HPC4 antibody recognition sequence, so we purified this fragment by affinity chromatography using the antibody column described before (20). The fraction bound to and eluted from the affinity chromatography column was concentrated by 10% trichloroacetic acid precipitation and fractionated by SDS-PAGE. The protein was transferred to a polyvinylidene fluoride membrane, and the C-terminal fragment was identified by Western blot using the HPC4 antibody. The N-terminal sequence of the peptide was determined at the Harvard Microchemistry Facility.

Site-directed Mutagenesis of Carboxylase—Expression and Purification of Carboxylase in Insect Cells—Oligonucleotides and PCR primers used for mutagenesis were synthesized by Invitrogen. Site-directed mutagenesis of cysteines of human γ-glutamyl carboxylase was conducted by the "Megaprimer" method of PCR mutagenesis (21, 22). Wild type carboxylase cDNA with a HPC4 tag at the C terminus (23) was used as template DNA for PCR. The mutations were screened by restriction digestion and verified by sequencing the entire cDNA. The wild type and cysteine mutants of carboxylase were expressed in High Five cells and purified as described previously (16). DTT was excluded from all of the purification steps, and the protease inhibition mixture was omitted from the sample destined for trypsin digestion. The active enzyme concentration was determined by fluorescence anisotropy titration of the enzyme against fluorescein-labeled consensus propeptide as described (18).

RESULTS

Inactivation of Carboxylase by Thiol-reactive Reagents—Because vitamin K-dependent carboxylase is inactivated by a variety of thiol-reactive reagents, free cysteines have been implicated as comprising part of the carboxylase active site (3, 8–10). Fig. 1 shows the inactivation curve of carboxylase by increasing concentrations of different thiol-reactive reagents at a fixed reaction time of 20 min at 0 °C. Na$_2$S$_4$O$_6$ and AMS (a hydrophilic maleimide derivative) inactivate carboxylase. In contrast, the hydrophobic maleimide derivative DABMI, which has a structure similar to AMS, does not inactivate carboxylase at concentrations up to 5 mM. These results suggest that cysteine residues that affect the enzyme activity by chemical modification may be located in the hydrophobic but not the hydrophilic regions of the carboxylase.

Hydrophilic Maleimide and Na$_2$S$_4$O$_6$ Modify the Same Cysteine Residues of Carboxylase—We next examined whether Na$_2$S$_4$O$_6$ and AMS inhibit carboxylase activity by reacting with the same cysteine residue(s). To accomplish this, carboxylase was first treated with Na$_2$S$_4$O$_6$, whose modification can be reversed by DTT. This triethylthionate enzyme was then treated with AMS. More than 90% of the carboxylase activity of Na$_2$S$_4$O$_6$-inactivated carboxylase was recovered after DTT treatment (Fig. 2). AMS-modified carboxylase is irreversibly inactivated. However, carboxylase first modified by Na$_2$S$_4$O$_6$ and then treated with AMS regains about 80% of its activity following DTT treatment. These results suggest that AMS and Na$_2$S$_4$O$_6$ modify the same cysteine residue(s) of the carboxylase.

Protection of Na$_2$S$_4$O$_6$ Inactivation of Carboxylase—If cysteine residues are part of the active site, then one might expect substrates to protect the enzyme from inactivation by thiol-reactive reagents. In addition to the glutamate substrate, γ-carboxylation requires VKH$_2$, carbon dioxide, and oxygen as co-substrates. Moreover, the propeptide of the Glu-containing substrate stimulates carboxylation (24). Our results (Table I) show that proFIX, proFIXgla, VKH$_2$, pentapeptide substrate (FLEEL), and different combinations of these substrates protect the carboxylase from inactivation by Na$_2$S$_4$O$_6$ to various extents.

It is noteworthy that proFIX and proFIXgla both significantly protect carboxylase from inactivation by Na$_2$S$_4$O$_6$, but...
carboxylase that has been nicked at residues 349 and 351 by limited trypsin digestion is less well protected, although still active. After 1 h of incubation with Na$_2$S$_4$O$_6$, intact carboxylase retains about 30% activity in the absence of proFIX, whereas in the presence of proFIX about 80% activity remains (Fig. 3 A). In contrast, the “nicked” carboxylase is not protected by the propeptide (Fig. 3 B).

Propeptide Binding Capacity of Intact and Nicked Carboxylase—One possible reason the nicked enzyme was not protected from Na$_2$S$_4$O$_6$ inactivation may be because it does not bind propeptide. To test this we compared the propeptide binding of the nicked enzyme to that of intact carboxylase using fluorescein-labeled proFIX. Our results show (Fig. 4 A) that the nicked carboxylase has binding characteristics similar to those of the intact carboxylase. The $K_d$ values for proFIX/intact carboxylase and proFIX/nicked carboxylase are 16.3 ± 0.4 and 37.3 ± 1 nM, respectively. Because we used a high concentration of propeptide in the protection experiments (8 µM), the enzyme should be saturated with substrate, and the loss of protection of propeptide against Na$_2$S$_4$O$_6$ inactivation of nicked carboxylase is not due to a lack of propeptide binding to the nicked enzyme. These results suggest that Na$_2$S$_4$O$_6$-modified cysteine residues are not located in the propeptide-binding site.

We further examined the propeptide binding capacities of Na$_2$S$_4$O$_6$-inactivated carboxylase using the fluorescein-labeled proCon. As shown in Fig. 4B, modification by Na$_2$S$_4$O$_6$ significantly decreases the affinity of carboxylase for the consensus propeptide ($K_d = 35$ versus 0.3 nM for unmodified). We observed a similar, although less dramatic, effect on the trypsin-nicked modified enzyme (Fig. 4 B).

Identification of AMS-modified Cysteine Residues of Carboxylase by MALDI-TOF MS—We employed MALDI-TOF MS to identify cysteine residues modified by the hydrophilic thiol-reactive reagent, AMS. The spectra for trypsin-digested modified and nonmodified samples are shown in Figs. 5 and 6. Compared with the nonmodified sample (Fig. 5, A, C, and E, and Fig. 6, A and C), we identified five major new peptide peaks ($m/z$ at 1603.63, 2409.13, 2537.22, 1915.77, and 2849.37) in the spectra of the AMS-modified sample (Fig. 5, B, D, and F, and Fig. 6, B and D). The new peptide peaks at $m/z$ 1630.63, 2409.13, and 2537.22 (Fig. 5, B, D, and F) represent AMS modification of Cys$^{323}$ containing tryptic peptides, KLVSC-PQKR (m/z at 1093.58), LVSYCPQRLQQLLPLK (m/z at 1899.09), and KLVSYCPQLQQLLPLK (m/z at 2027.18), re-
respectively. The peptide peaks at m/z 1915.77 and 2849.37 (Fig. 6, B and D) derive from AMS modification of Cys343-containing tryptic peptides AAPQPSVSCVYKR (m/z at 1405.73) and LQQQLPLKAAPQPSVSCVYKR (m/z at 2339.33), respectively. The skipped trypsin cleavage sites are identified in bold type. All 10 cysteines of the carboxylase were identified, and Cys323 and Cys343 are the only two cysteines modified by AMS under our reaction conditions. The mass increase from AMS modification of a cysteine-containing tryptic peptide is 510 Da, which is 18 Da more than the expected mass increase due to the addition of AMS. This 18-Da mass difference arises from the opening of the maleimide ring through hydrolysis as reported previously (25).

None of the five new peptide peaks from AMS modification were present when carboxylase was treated with Na2S4O6 before AMS modification (data not shown). This confirms the results shown in Fig. 2 indicating that AMS and Na2S4O6 react at the same sites.

**MS/MS Analysis of AMS-modified Cysteine-containing Tryptic Peptides**—To confirm the identities of the peaks assigned by MALDI-TOF MS described in the previous section, we isolated the new peptides at m/z 1603.63 and 1915.77 using a timed ion selector. The selected peptides were subjected to collision-induced dissociation for amino acid sequencing. The most commonly observed product ions resulting from collision-induced dissociation belong to the y-type ion series, which results from backbone cleavage of the C–N amide linkage with the charge retained on the C-terminal fragments. The product ions are numbered according to the cleavage site from the C-terminal end. The fragmentation spectrum of the peptide ion at m/z 1603.63 revealed a series of y-ions from the tryptic peptide KLVSYCPQR containing Cys323 (Fig. 7A). The fragmentation of peptide ion at m/z 1915.77 produces a series of y-ions from the tryptic peptide AAPQPSVSCVYKR containing Cys343 (Fig. 7B). There is a large gap between the y3 and y4 ions in Fig. 7A and the y4 and y5 ions in Fig. 7B. This represents AMS modification of the Cys323 and Cys343 at the fourth and fifth positions of the corresponding tryptic peptide from the C terminus. Because of AMS modification, all of the y-ions containing the cysteine residue have a 510-Da mass increase compared with the theoretical mass of the corresponding y-ions of the peptide with an unmodified cysteine residue. These results further confirm that Cys323 and Cys343 are the target cysteine residues for AMS modification.
Localization of Cys\textsuperscript{323} and Cys\textsuperscript{343} with Respect to the Endoplasmic Reticulum Membrane—Our previous results indicate that Cys\textsuperscript{323} and Cys\textsuperscript{343} are in the cytoplasm (20), but the active site residues are apparently in the lumen of the endoplasmic reticulum (26, 27). To confirm our previous membrane topology and determine where Cys\textsuperscript{323} and Cys\textsuperscript{343} are located relative to the membrane, we isolated the C-terminal peptide of the carboxylase by treating intact carboxylase containing microsomes with proteinase K and purifying the protected (lumenal) fragment with the HPC4 antibody tag. Harvard Microchemistry...
We used site-directed mutagenesis to mutate individual cysteine residues in carboxylase. Mutant proteins were expressed in insect cells, and the expressed proteins were purified by affinity chromatography. Purified carboxylase activity was assayed by the incorporation of $^{14}$CO$_2$ into the substrate FLEEL. The concentration of active carboxylase was determined from the fraction of protein binding to the propeptide (18).

| Carboxylase | Specific activity (mean ± S.D., n = 3) | Activity |
|-------------|---------------------------------------|----------|
| Wild type   | 1192 ± 17                             | 100      |
| C323S       | 1144 ± 24                             | 96       |
| C323A       | 1740 ± 6                              | 146      |
| C343S       | 1240 ± 31                             | 104      |
| C343A       | 447 ± 2                              | 38       |
| 6-Cys-mut   | 1076 ± 20                             | 90       |
| C288A       | 1235 ± 30                             | 104      |
| C288S       | 667 ± 13                              | 56       |

Facility identified the N-terminal sequence of the purified fragment as SGQKPGLRHQ, corresponding to the sequence of carboxylase starting from amino acid residue 352. This result agrees with our previous report on the membrane topology of carboxylase (20). For our interpretation to be wrong there would have to be two additional transmembrane domains between residues 313 and 360. Because this sequence is hydrophilic in nature this possibility seems unlikely. Thus Cys$^{223}$ and Cys$^{343}$ must reside in the cytoplasm, because there is no other plausible transmembrane domain in this region.

Site-directed Mutagenesis of Cysteine Residues in Carboxylase—We used site-directed mutagenesis to mutate individual or multiple cysteine residues in carboxylase. Mutant proteins were expressed and affinity-purified from insect cells. The specific activity of each mutant enzyme was determined (18) and compared with that of the wild type carboxylase. Neither alanine nor serine mutants of Cys$^{223}$ exhibit decreased carboxylase activity (Table II). In fact, the C323A mutant carboxylase has higher specific activity than the wild type enzyme. The C343S mutant carboxylase is fully active, whereas the C343A has 38% specific activity. These results indicate that Cys$^{223}$ and Cys$^{343}$ are not necessary for carboxylase activity. Carboxylase with the following six cysteine mutations in combination, C134W, C139S, C311S, C323S, C598A, and C700A, retains 90% of its normal activity. However, if one additional free cysteine is mutated, either Cys$^{288}$ or Cys$^{313}$, the enzyme activity drops to 0 (data not shown).

**DISCUSSION**

We previously demonstrated that Cys$^{99}$ and Cys$^{450}$ form the only disulfide bond in vitamin K-dependent carboxylase. In addition, we suggested, based upon the results of that study, that cysteine residues are not directly involved in the catalytic steps of carboxylation (15). Because it is clear from our work and that of others that chemical modification of cysteine residues inactivates carboxylase, in the present study we attempted to clarify the role of cysteines in carboxylase. We extended our previous work by using different types of reagents for chemical modification and did additional site-directed mutagenesis to cysteines, using alanine instead of serine substitutions to eliminate the possibility that serines can participate in carboxylation. We determined the residues modified by sodium tetrathionate and AMS, two different sulfhydryl reagents that inactivate carboxylase, and attempted to determine why activity is affected. We examined the kinetics of inactivation and the effect on catalysis and propeptide binding. In addition we identified the residues modified using MALDI-TOF mass spectrometry. Finally we performed chemical modifications of mutated carboxylase to identify which of the modified residues were responsible for activity loss.

In the present study we tested the effects of several thiol-reactive reagents on carboxylase to compare them to our previous work. As shown in Fig. 1, carboxylase activity was significantly inhibited by the hydrophilic maleimide derivative AMS but not by the hydrophobic derivative DABMI. The results from the present study using mass spectrometry showed that the hydrophilic maleimide derivative AMS modified only Cys$^{232}$ and Cys$^{343}$. Our earlier results (13) show that N-ethylmaleimide modifies Cys$^{139}$, Cys$^{311}$, Cys$^{323}$, and Cys$^{343}$ of carboxylase. The larger AMS may have less access to cysteines because of steric issues and thus modified only two residues as opposed to the smaller reagent, NEM.

Modification by Na$_2$S$_4$O$_6$ (Fig. 3) also inactivated carboxylase. The time course of inactivation was consistent with modification of more than one residue because the data was best fit by a two exponential equation. The Na$_2$S$_4$O$_6$ reaction has the advantage of ready reversibility by mild reducing agents, such as DTT (28). The reversible inactivation of carboxylase by Na$_2$S$_4$O$_6$ provided an opportunity to determine whether the maleimide derivative, AMS, and Na$_2$S$_4$O$_6$ reacted with the same cysteine residue(s). We sequentially treated carboxylase with Na$_2$S$_4$O$_6$ and AMS. If AMS targets the same cysteine as Na$_2$S$_4$O$_6$, then the tetrathionated cysteine would no longer be accessible to AMS, and the carboxylase activity would be recovered with DTT. If AMS targeted a different cysteine that results in the inactivation of the enzyme, then the enzyme activity would not be recovered by DTT because the modification of cysteines by AMS is irreversible. As shown in Fig. 2, the activity of Na$_2$S$_4$O$_6$-inactivated carboxylase further modified by AMS was recovered after reaction with the reducing reagent DTT. This indicates that inactivation of carboxylase by AMS and Na$_2$S$_4$O$_6$ was caused by modification of the same cysteine residue(s). We further confirmed this interpretation because the MALDI-TOF spectrum of the AMS-modified Na$_2$S$_4$O$_6$-inactivated carboxylase revealed no AMS-labeled tryptic peptides (data not shown). This indicates that modification of Cys$^{223}$ and/or Cys$^{343}$ is sufficient for carboxylase inactivation. In addition, when we modified either of the fully active C323S or C343S mutant carboxylases with Na$_2$S$_4$O$_6$, the enzymes were inactivated (data not shown). This suggests that modification of either of these two cysteines will inactivate the enzyme.

It is possible that inactivation of carboxylase by chemical modification is caused by reaction with a residue other than cysteine. We identified 15 of the 27 lysine residues in the mass spectrum of AMS-modified carboxylase. None were modified, but we cannot rule out a possible lysine modification. However, under the conditions we used, the rate of maleimide reaction with cysteine thiols is ~1000-fold faster than with amino groups of lysine (29). Another argument against a spurious modification is that we and others have shown that carboxylase can be inactivated by a variety of thiol-reactive agents (3, 8–11). It seems unlikely that all of these reagents would modify a functionally important residue other than cysteine.

The active site of carboxylase is presumably in the endoplasmic reticulum lumen (26, 27). Our published membrane topology of carboxylase (20) demonstrates five transmembrane domains and indicates that Cys$^{322}$ and Cys$^{343}$, the AMS-modified cysteines, are located on the cytoplasmic side of the endoplasmic reticulum membrane (Fig. 8B). In the present study, to confirm that Cys$^{323}$ and Cys$^{343}$ are in the cytoplasm, we identified the N terminus of the last transmembrane domain of carboxylase. Consistent with our previous report, we found...
that Ser^352 is the N-terminal residue of the last transmembrane domain, so it is unlikely that either Cys^323 or Cys^343 is in the lumen.

In our previous study we made carboxylases with mutations of each of the 10 cysteine residues, most to serine, including Cys^323 and Cys^343. To further investigate the role of cysteine in activity, we mutated Cys^323 and Cys^343 to alanine. In addition we mutated Cys^288 to alanine because its serine mutation had slightly reduced activity as reported in our earlier paper (13). We substituted alanine instead of serine for cysteine to rule out the possibility that serine can function in carboxylation. Although the activities of C343S, C323A, or C323S mutant carboxylases were normal, C343A had only 38% activity (Table II). Still it seems unlikely that these two residues are at the active site of carboxylase. The 60% reduction in C343A activity may indicate that an important hydrogen bond is disrupted when alanine is substituted for cysteine, but not when serine is substituted.

Because single cysteine mutations had little effect on activity, we made carboxylase with multiple mutations: C134W, C139S, C311S, C323S, C598A, and C700A. As with the carboxylase that had these mutations individually, the carboxylase with the six-cysteine mutation had activity similar to wild type enzyme (Table II). However, mutation of any one additional cysteine residue (Cys^99, Cys^288, Cys^343, or Cys^450) caused a total loss of enzyme activity (data not shown). The effect of adding Cys^99 or Cys^450 to the combined mutation is easy to understand, because we know from our previous work that Cys^99 and Cys^450 are involved in the single disulfide bond of carboxylase (13). However, the effect of adding Cys^288 or Cys^343 to the combined mutation is less easily understood. A work published during review of our study reports that carboxylase...
with all 10 cysteines changed to alanine has low levels of residual activity when co-expressed with factor IX but otherwise supports our conclusions about the role of cysteines in catalysis (14).

So why does chemical modification of cysteine knock out carboxylase activity, but mutation does not? Unfortunately the chemically modified enzyme has so little activity that we could not perform kinetic analyses to compare modified and mutant enzymes directly. However, we did measure propeptide binding and the effect of substrates on rates of inactivation by thiol-reactive reagents. The results showed that the most effective protection from Na2S4O6 inactivation came from either proFIX or the propeptide-containing substrate, proFIXgla (Table I). Consistent with protection by the propeptide, the chemically modified carboxylase had reduced affinity for propeptide compared with that of the native enzyme (Fig. 4B). This does not appear to be due to a direct effect on the propeptide binding site because the cysteine mutant carboxylases all bound propeptide with affinity similar to that of wild type. In addition, trypsin-nicked carboxylase is not protected from chemical modification (Fig. 3B), but this enzyme binds propeptide with normal affinity (Fig. 4).

These results together suggest that the Na2S4O6-modified cysteine is not directly involved in propeptide binding but affects activity by interruption of the secondary and/or tertiary structure of carboxylase. This is manifested, not only in a loss of activity, but also in a loss of affinity for the propeptide of the substrate. This conclusion is consistent with our previous study showing a relationship between loss of enzyme activity and propeptide binding in which we showed that heat-inactivated (presumably denatured) carboxylase does not bind to propeptide (18).

In conclusion, we examined the role of free cysteine residues of vitamin K-dependent carboxylation using chemical modification and site-directed mutagenesis. Chemically modified cysteine residues were identified by MALDI-TOF MS. The results show that modification of Cys323 and/or Cys343 by hydrophilic thiol-reactive reagents inactivates the carboxylase, whereas carboxylase mutants of these two residues either are fully active or have modestly decreased activities. The effect of chemical modification on propeptide binding to intact and trypsin-nicked carboxylase and sodium tetrathionate-inactivated intact and nicked carboxylase suggests that inactivation of carboxylase by chemical modification is due to the disruption of the enzyme structure rather than modification of active site cysteine residues. Therefore, we conclude that cysteine residues are not directly involved in the chemical reactions essential to vitamin K-dependent carboxylation.

Acknowledgments—We thank Chun-Yun Chang and Pen-Jin Lin for the excellent technical assistance. We also thank Dr. Scott Whitney (Invitrogen) for helpful discussions.

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Chemical Modification of Cysteine Residues Is a Misleading Indicator of Their Status as Active Site Residues in the Vitamin K-dependent γ-Glutamyl Carboxylation Reaction

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J. Biol. Chem. 2004, 279:54079-54087.
doi: 10.1074/jbc.M408945200 originally published online October 18, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M408945200

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