Glutamyl Substrate-Induced Exposure of a Free Cysteine Residue in the Vitamin K-Dependent γ-Glutamyl Carboxylase Is Critical for Vitamin K Epoxidation†

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ABSTRACT: The vitamin K-dependent carboxylase catalyzes the posttranslational modification of glutamic acid to γ-carboxyglutamic acid in the vitamin K-dependent proteins of blood and bone. The vitamin K-dependent carboxylase also catalyzes the epoxidation of vitamin K hydroquinone, an obligatory step in γ-carboxylation. Using recombinant vitamin K-dependent carboxylase, purified in the absence of propeptide and glutamic acid-containing substrate using a FLAG epitope tag, the role of free cysteine residues in these reactions was examined. Incubation of the vitamin K-dependent carboxylase with the sulfhydryl-reactive reagent *N*-ethylmaleimide inhibited both the carboxylase and epoxidase activities of the enzyme. This inhibition was proportional to the incorporation of radiolabeled *N*-ethylmaleimide. Stoichiometric analyses using [^{3}H]-*N*-ethylmaleimide indicated that the vitamin K-dependent carboxylase contains two or three free cysteine residues. Incubation with propeptide, glutamic acid-containing substrate, and vitamin K hydroquinone, alone or in combination, indicated that the binding of a glutamic acid-containing substrate to the carboxylase makes accessible a free cysteine residue that is important for interaction with vitamin K hydroquinone. This is consistent with our previous observation that binding of a glutamic acid-containing substrate activates vitamin K epoxidation and supports the hypothesis that binding of the carboxylatable substrate to the enzyme results in a conformational change which renders the enzyme catalytically competent.

The vitamin K-dependent carboxylase (carboxylase)[1] catalyzes the posttranslational modification of specific glutamic acid residues to γ-carboxyglutamic acid (Gla) in the vitamin K-dependent proteins of blood *(1)*. These include the coagulation proteins factor VII, factor IX, factor X, and prothrombin, and the blood anticoagulants protein C and protein S. Gla residues confer metal-binding properties on these proteins *(2)*. In the presence of metal ions, these proteins express membrane-binding properties that are essential for their participation in blood clotting *(3−7)*. In addition to the precursor forms of the vitamin K-dependent proteins of blood, other known Gla-containing proteins include two bone proteins, osteocalcin *(8, 9)* and matrix Gla protein *(10)*, which appear to have roles in regulation of tissue mineralization *(11, 12)*; Gas6, a putative ligand for receptor tyrosine kinases *(13)*; and PRGP1 and PRGP2, two newly discovered proline-rich Gla-containing proteins of unknown function *(14)*. All of the known protein substrates of carboxylase possess a well-conserved recognition sequence that directs γ-carboxylation. The γ-carboxylation recognition sequence is usually within the propeptide of the substrate *(15)*.

The conversion of glutamic acid to γ-carboxyglutamic acid by the carboxylase requires vitamin K hydroquinone (vitamin KH_2), CO_2, and O_2 *(1)*. In this reaction, the γ-proton on glutamic acid is abstracted followed by addition of CO_2 (Figure 1). It has been hypothesized that an active oxygenated species of vitamin K abstracts a hydrogen from the γ-carbon of glutamic acid, with subsequent collapse of the activated vitamin K species to vitamin K epoxide. Carbon dioxide is subsequently added to the γ-carbon of glutamic acid. Based upon a nonenzymatic model *(16)*, a "base strength amplification mechanism" has been proposed to explain the conversion of vitamin KH_2 into an oxygenated intermediate of sufficient basicity to abstract a hydrogen from the γ-carbon of glutamic acid *(17)*. Since the oxidation of reduced vitamin K precedes reaction at the γ-C-H on the substrate glutamic acid residue, it is critical that the short-lived highly reactive vitamin K intermediate not be generated intracellularly in the absence of substrate glutamic acid residues. Indeed, it has been recently demonstrated that, like γ-carboxylation, oxygenation of vitamin K by the carboxylase does not occur in the absence of propeptide or glutamic acid-containing substrate

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[1] Abbreviations: carboxylase, vitamin K-dependent carboxylase; Gla, γ-carboxyglutamic acid; vitamin KH_2, vitamin K hydroquinone; DTT, dithiothreitol; *p*HMB, *p*-hydroxymercuribenzoic acid; NEM, *N*-ethylmaleimide; IA, iodoacetic acid; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; [^{3}H]-NEM, *N*-[*ethyl*-1,2-^{3}H]-maleimide; proPT18, HVFLAPQQARSLLQRVRR; proPT28, HVFLAPQQARSLLQRVRRANTFLEEVRK; FLAG-carboxylase, recombinant, FLAG epitope-tagged, bovine vitamin K-dependent carboxylase; PBS/Tween, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3, containing 0.05% Tween 20.

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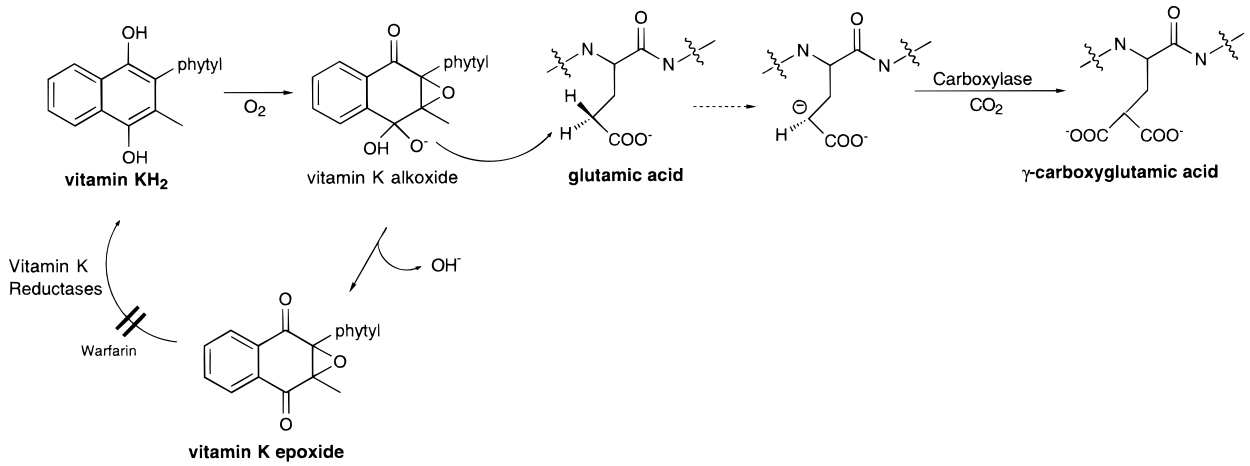

FIGURE 1: Reaction scheme for formation of γ-carboxyglutamic acid and vitamin 2,3-epoxide by the vitamin K-dependent carboxylase. In this scheme, vitamin K alkoxide is the hypothetical very strong base which abstracts a proton from the γ-C-H of glutamic acid. The activated vitamin K species collapses to vitamin K 2,3-epoxide. Vitamin K 2,3-epoxide is recycled back to vitamin KH_2 by the action of two vitamin K reductases, one of which is sensitive to warfarin.

(18). Upon the addition of propeptide or substrate, the enzyme is converted to an active epoxidase. Therefore, no highly reactive vitamin K intermediate is generated until binding of a carboxylatable substrate activates the enzyme system.

Various studies using the reducing agent dithiothreitol (DTT) and/or sulfhydryl-reactive reagents have suggested a role for free cysteine residues in function of the carboxylase *(19−24)*. The addition of DTT to crude bovine liver enzyme preparations was shown to enhance γ-carboxylation 1.5−3-fold *(19−22)* while sulfhydryl reactive reagents such as *p*-hydroxymercuribenzoic acid (*p*HMB), *N*-ethylmaleimide (NEM), and iodoacetic acid (IA) were inhibitory *(20−23)*. *p*HMB and NEM inhibit epoxidase activity of the vitamin K-dependent carboxylase as well *(23)*. More recently, the effect of NEM on carboxylase purified from bovine liver using propeptide-affinity chromatography was examined *(24)*. Incubation with NEM inhibited carboxylase activity by 60−70%. Based on these collective observations, Naganathan et al. proposed a role for free cysteine residues in the mechanism of the vitamin K-dependent carboxylase *(25)*. The schema includes a free thiol sufficiently basic to abstract a proton from vitamin K hydroquinone, initiating the oxygenation that leads to a vitamin K species of sufficient basicity to abstract a γ-proton from a glutamate residue. In the current study, the role of free cysteine residues in the enzyme activities of purified, recombinant, FLAG epitope-tagged bovine carboxylase was examined. The results indicate that the vitamin K-dependent carboxylase contains at least one free cysteine residue which is "exposed" upon binding of an appropriate substrate to the carboxylase. This cysteine residue appears to be involved in an interaction with vitamin KH_2.

EXPERIMENTAL PROCEDURES

Materials. N-[*ethyl*-1,2-^{3}H]Maleimide ([^{3}H]-NEM) (60 Ci/mmol) was from Amersham. The synthetic peptides FLEEL, proPT18 (HVFLAPQQARSLLQRVRR, a peptide representing residues −18 to −1 of proprothrombin), and proPT28 (HVFLAPQQARSLLQRVRRANTFLEEVRK, a peptide representing residues −18 to +10 of acarboxy-proprothrombin) were synthesized as described previously *(18)*.

Recombinant, FLAG epitope-tagged, bovine vitamin K-dependent carboxylase (FLAG-carboxylase) was prepared as described in detail previously *(26)*. The enzyme is >90% homogeneous by SDS−PAGE and silver staining. It exhibits a specific activity and kinetic constants that are comparable to those for native bovine liver carboxylase *(27)*.

Vitamin K-Dependent Carboxylase Quantitation. The concentration of a stock FLAG-carboxylase standard was determined using a quantitative Western blot described previously *(27)*. Aliquots of this FLAG-carboxylase standard were stored at −80 °C for use in an ELISA.

The concentrations of FLAG-carboxylase samples were determined by ELISA. Each well of a microtiter plate (Linbro E. I. A. II Plus Microtitration plate, Flow Laboratories Inc., McLean, VA) was coated with 50 μL of sample or FLAG-carboxylase standard (2, 1.5, 1, 0.75, 0.5, 0.325, 0.25, 0.1, and 0 μg/mL in 10 mM sodium phosphate, pH 7.1) at 4 °C overnight. After incubation, the excess FLAG-carboxylase was removed, and the nonspecific binding sites were blocked with 250 μL of 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3 (PBS), containing 0.05% Tween 20 (PBS/Tween), and 5% nonfat milk for 1 h at 24 °C. The wells were washed 3 times with PBS/Tween. Bound FLAG-carboxylase was detected by 100 μL of a rabbit anti-carboxylase peptide antibody (20 μg/mL in PBS/Tween). This antibody is directed against a synthetic peptide (SSLDRRYLDGLEVC) representing amino acids 86−99 of bovine carboxylase and has been described previously *(28)*. Following incubation for 1 h at 24 °C, the wells were washed 3 times with PBS/Tween. An anti-rabbit alkaline phosphatase-conjugated antibody (1:2500 dilution in PBS/Tween, 100 μL/well) was used to detect the anti-carboxylase peptide antibody. Following a 45 min incubation at 24 °C, the wells were washed 5 times with PBS/Tween. Two hundred microliters of substrate (1 mg/mL *p*-nitrophenyl phosphate in 50 mM sodium carbonate, 0.5 mM $MgCl_2$, pH 9.6) was added to each well. The reaction was allowed to proceed for 45 min and the product detected at a wavelength of 405 nm. In each experiment, the absorbance of the unknown samples was compared to a standard curve generated using the stock FLAG-carboxylase.

Assay for Vitamin K-Dependent Carboxylase and Epoxidase Activities. Vitamin K_1 (10 mg/mL), purchased from Abbott, was chemically reduced with 8 mg of $NaBH_4$ for 30 min at ambient temperature under a N_2 atmosphere protected from light. It was diluted 1:4 with PBS/0.1% CHAPS prior to use. Carboxylase activity was measured as the incorporation of ^{14}C-labeled CO_2 into a synthetic peptide substrate (FLEEL) in the presence of propeptide as described previously with the exception that DTT was omitted from the reaction mixture *(18)*. Vitamin K 2,3-epoxide formation in the presence of both FLEEL and propeptide was measured as described previously *(18)*. During the measurement of both activities, background values were obtained by performing reactions in the absence of vitamin KH_2 or FLAG-carboxylase.

Inhibition of Vitamin K-Dependent Carboxylase Activity with Iodoacetic Acid and p-Hydroxymercuribenzoic acid. FLAG-carboxylase was incubated with either IA (0.25−10 mM in 0.1 N NaOH) or *p*HMB (0.05−1 mM in 6 M guanidine hydrochloride/0.2 M Tris, pH 8.6) for 30 min at 0 °C. Carboxylase activity was measured as described above. Inhibition by either IA or *p*HMB was compared to the effect of incubation with vehicle alone.

Inhibition of Enzyme Activity with N-Ethylmaleimide. FLAG-carboxylase was incubated with varying concentrations of NEM (0.5−20 mM in ethanol) for 30 min at 0 °C, or for 30 min at 24 °C. In subsequent experiments, FLAG-carboxylase was labeled with 5 mM NEM at 24 °C. Twenty microliter aliquots of the reaction were removed over time (0−30 min), and quenched by dilution into 30 μL of PBS containing DTT (40 mM final concentration) and freezing at −80 °C. Vitamin K-dependent epoxidation and carboxylation were measured as described above. Parallel experiments were performed to determine the effects of incubation of FLAG-carboxylase with vehicle alone.

Incorporation of [^{3}H]-N-Ethylmaleimide. FLAG-carboxylase was incubated with NEM as before except that a 1:5 mixture of [^{3}H]-NEM and unlabeled NEM (5 mM final concentration of ^{3}H-labeled plus unlabeled NEM) was used. Twenty microliter aliquots of the reaction were removed over time (0−30 min), diluted into 30 μL of PBS containing DTT (40 mM final concentration), and frozen at −80 °C. The free NEM was removed by extensive dialysis against PBS containing 0.125% CHAPS. The [^{3}H]-NEM that was incorporated into free cysteines was quantitated by counting the retentate and the dialysis bag together using a β-counter after the addition of 5 mL of Atomlight scintillation fluid. The data are expressed as the total counts for each sample subtracted from the counts incorporated at 0 min.

Stoichiometric Analyses. FLAG-carboxylase was labeled with [^{3}H]-NEM for 30 min at ambient temperature as described above. Following dialysis to remove free [^{3}H]-NEM, an aliquot of the retentate was counted on a β-counter after the addition of 5 mL of Atomlight scintillation fluid, and the moles of NEM incorporated per 1 μL of the reaction were determined. Using a second sample of the same size, the moles of FLAG-carboxylase per 1 μL of the reaction were determined by ELISA as described above. The results of these two quantitations were compared to determine the number of free cysteine residues that are labeled by NEM per mole of carboxylase.

Effects of Propeptide, Glutamic Acid-Containing Substrate, and Vitamin KH_2 on Incorporation of NEM into Carboxylase. To assess the effect of propeptide on NEM incorporation, FLAG-carboxylase was incubated with either proPT18 (10 μM) or proPT28 (10 μM) for 30 min at 0 °C. To assess the effect of glutamic acid-containing substrate, FLAG-carboxylase was incubated with either proPT28 (10 μM); FLEEL (5 or 10 mM); or proPT18 (10 μM) and FLEEL (10 mM) for 30 min at 0 °C. In control reactions, FLAG-carboxylase was incubated with buffer alone. After incubation with propeptide and/or substrate, FLAG-carboxylase was labeled with a 1:5 mixture of [^{3}H]-NEM and unlabeled NEM (0.5 mM final concentration) at 0 °C. The incorporation of NEM over time (0−30 min) was determined as described. NEM incorporation at 30 min for carboxylase incubated with propeptide and/or substrate is expressed as a percentage of that for carboxylase incubated with buffer alone.

In other experiments FLAG-carboxylase was incubated with proPT18 (10 μM) and vitamin KH_2 (222 μM); proPT28 (10 μM) and vitamin KH_2 (222 μM); FLEEL (5 mM) and vitamin KH_2 (222 μM); or proPT18 (10 μM), FLEEL (5 mM), and vitamin KH_2 (222 μM). In these experiments, FLAG-carboxylase was incubated first with propeptide and/or glutamic acid-containing substrate for 15 min at 0 °C followed by the addition of and incubation with vitamin KH_2 for an additional 15 min at 0 °C. FLAG-carboxylase was also incubated with vitamin KH_2 alone (222 μM) for 30 min at 0 °C. In control reactions, vitamin KH_2 was omitted or FLAG-carboxylase was incubated with buffer alone. After incubation, FLAG-carboxylase was labeled with a 1:5 mixture of [^{3}H]-NEM and unlabeled NEM (5 mM final concentration) at 24 °C. The incorporation of NEM over time (0−30 min) was determined as described. NEM incorporation at 20 min for reactions performed in the presence of propeptide, substrate, and/or vitamin KH_2 is expressed as a percentage of that for carboxylase incubated with propeptide and/or substrate alone, or carboxylase incubated with buffer alone.

RESULTS

In agreement with previous observations *(20−24)*, sulfhydryl-reactive reagents, *p*HMB, IA, and NEM, inhibited our FLAG epitope-tagged vitamin K-dependent carboxylase. Under conditions in which these reagents react specifically with free cysteine residues, both *p*HMB and IA inhibited γ-carboxylation of a synthetic substrate, FLEEL, in a concentration-dependent manner (data not shown). Incubation with stoichiometric concentrations of DTT blocked the effect of *p*HMB. Incubation of FLAG-carboxylase with NEM also inhibited γ-carboxylation of FLEEL. Experiments were performed to assess the concentration, time, and temperature dependence of the reaction with NEM. After a 30 min incubation at 0 °C, ∼75% of the initial carboxylase activity was inhibited at an NEM concentration of 20 mM (Figure 2). When the incubation was performed for 30 min at 24 °C, maximal inhibition of carboxylase activity (≥98%) was observed after 20 min at an NEM concentration of 5 mM (Figure 2). Thus, to ensure we were achieving maximal inhibition, FLAG-carboxylase was incubated with 5 mM NEM at 24 °C for 30 min in subsequent experiments.

Since the oxygenation of vitamin KH_2 is an obligatory step in γ-carboxyglutamic acid synthesis (see Figure 1), we

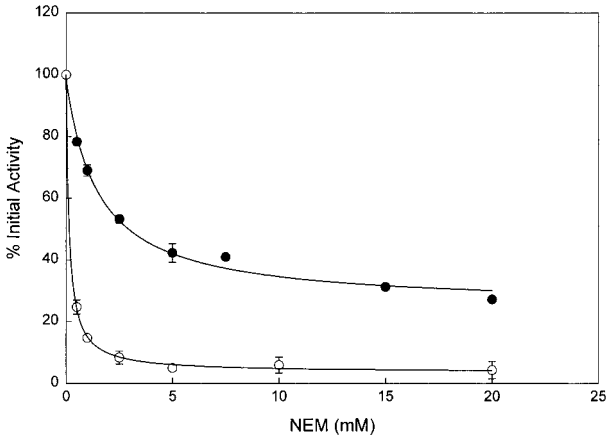

FIGURE 2: Inhibition of in vitro γ-carboxylation of a synthetic substrate by the vitamin K-dependent carboxylase following incubation with NEM. FLAG-carboxylase was incubated with increasing concentrations of NEM (0.5−20 mM) for either 30 min at 0 °C (●) or 30 min at 24 °C (○). Vitamin K-dependent carboxylase activity was assayed as described under Experimental Procedures. The percent inhibition was determined by comparing the amount of $^{14}CO_2$ incorporation into FLEEL by NEM-treated carboxylase to that for enzyme incubated in the absence of NEM. The data that are shown represent the mean ± sem for one of three identical experiments.

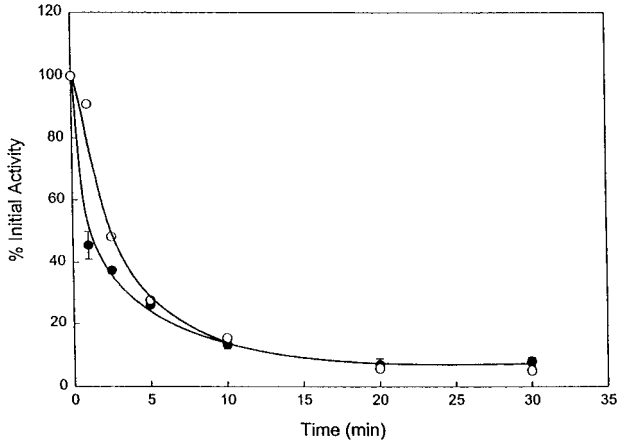

FIGURE 3: Inhibition of the carboxylase and epoxidase activities of the vitamin K-dependent carboxylase by NEM. FLAG-carboxylase was incubated with 5 mM NEM at 24 °C for 30 min. Aliquots were removed at the times indicated for assay of carboxylation of FLEEL in a vitamin KH_2-dependent manner as described under Experimental Procedures. Similarly, aliquots were removed and assayed for formation of vitamin K 2,3-epoxide as described under Experimental Procedures. The percent inhibition of carboxylation (●) or epoxidation (○) was determined by comparison to the values obtained by enzyme incubated in the absence of NEM. The data that are shown represent the mean ± sem for one of two identical experiments.

determined whether NEM inhibited vitamin K epoxidation by the carboxylase. Similar to our observation for carboxylation of FLEEL, incubation of FLAG-carboxylase with NEM inhibited epoxidation of vitamin KH_2 in a time-dependent manner with maximal inhibition occurring after a 20 min incubation with 5 mM NEM at 24 °C (Figure 3). The initial rate of NEM inhibition of carboxylation was faster than the initial rate of NEM inhibition of epoxidation, suggesting that the carboxylase active site contains more than one thiol with differing susceptibilities to derivatization by NEM.

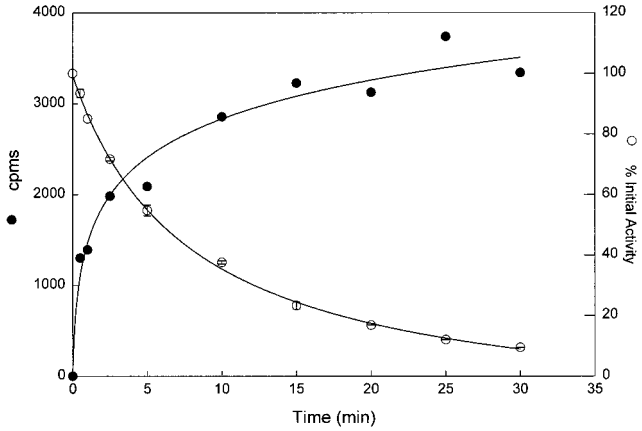

FIGURE 4: Incorporation of [^{3}H]-NEM versus carboxylase activity following treatment with NEM as a function of time. FLAG-carboxylase was incubated with 5 mM of a 1:5 mixture of [^{3}H]-NEM and unlabeled NEM. Free NEM was removed by dialysis, and the bound [^{3}H]-NEM was quantitated using a β-counter after the addition of 5 mL of Atomlight scintillation fluid. The data are expressed as the total counts for each sample subtracted from the counts incorporated at 0 min (●). In these experiments, the amount of error associated with each time point ranged from ∼1.2 to 5% of the mean value. FLAG-carboxylase was incubated with 5 mM NEM at 24 °C over time (0−30 min). Vitamin K-dependent carboxylase activity was measured as described. The percent inhibition (○) was determined by comparing the amount of $^{14}CO_2$ incorporation into FLEEL by NEM-treated carboxylase to that for enzyme incubated in the absence of NEM. The data that are shown represent the mean ± sem for one of two identical experiments.

The incorporation of NEM into FLAG-carboxylase was compared to its ability to inhibit carboxylase activity in the same experiment. [^{3}H]-NEM was incorporated into the FLAG-carboxylase in a time-dependent manner (Figure 4). Half-maximal incorporation of NEM into FLAG-carboxylase occurred after a ∼2 min incubation while half-maximal inhibition of carboxylase activity was at ∼5 min (Figure 4), suggesting that the cysteine residue(s) derivatized most rapidly may not be the one(s) important for catalysis. The number of free cysteine residues that were derivatized by NEM was determined by stoichiometric analyses. In four independent determinations, the moles of [^{3}H]-NEM that were incorporated into the carboxylase were directly compared to the moles of FLAG-carboxylase determined by ELISA. These analyses demonstrated that 2.5 ± 0.5 mol of NEM was incorporated per mole of carboxylase. This indicates that the vitamin K-dependent carboxylase contains 2−3 free cysteine residues. Furthermore, the combined data indicate that at least one of these free cysteine residues is involved in enzyme activity.

The effect of propeptide and glutamic acid-containing substrate on NEM incorporation was examined. In contrast to carboxylase purified by affinity chromatography using propeptide *(29, 30)*, FLAG-carboxylase preparations are not exposed to propeptide or glutamic acid-containing substrate. Furthermore, the addition of the FLAG epitope to the N-terminus of the vitamin K-dependent carboxylase has no effect on the carboxylase and epoxidase activities of the enzyme, and the FLAG-carboxylase is not altered by the purification method employed *(18)*. Thus, the availability of FLAG-carboxylase preparations free of contaminating propeptide or glutamic acid-containing substrate allows for the study of the effects of propeptide and substrate on NEM incorporation. In these experiments, FLAG-carboxylase was incubated

Table 1: Effects of Propeptide and/or Glutamic Acid-Containing Substrate on the Incorporation of NEM into the Vitamin K-Dependent Carboxylase

addition	% control[a,b]
proPT18 (10 μM)	100 ± 2
proPT28 (10 μM)	140 ± 12
FLEEL (10 mM)	220 ± 19
proPT18 (10 μM) plus FLEEL (10 mM)	170 ± 4

[a] The data represent the mean ± sem of two or more experiments. [b] NEM incorporation expressed as a percentage of that observed in the presence of buffer alone.

Table 2: Effect of Vitamin KH_2 on the Incorporation of NEM into the Vitamin K-Dependent Carboxylase

addition	% control[a,b]
vitamin KH_2 (222 μM)	100 ± 8
proPT18 (10 μM) plus vitamin KH_2 (222 μM)	97 ± 6
proPT28 (10 μM) plus vitamin KH_2 (222 μM)	59 ± 0
FLEEL (5 mM) plus vitamin KH_2 (222 μM)	56 ± 2
proPT18 (10 μM), FLEEL (5 mM), vitamin KH_2 (222 μM)	49 ± 7

[a] The data represent the mean ± sem of two or more experiments. [b] NEM incorporation expressed as a percentage of that observed in the absence of vitamin KH_2.

with NEM under conditions that result in incomplete incorporation of NEM into the carboxylase (0.5 mM NEM, 0 °C). Incubation of FLAG-carboxylase with a synthetic peptide with the sequence of residues −18 to −1 of proprothrombin, proPT18 (HVFLAPQQARSLLQRVRR), had no effect on the incorporation of NEM measured at 30 min after addition of NEM (100 ± 2%) (Table 1). In contrast, incubation of FLAG-carboxylase with the synthetic carboxylase substrate proPT28 (HVFLAPQQARSLLQRVRRANTFLEEVRK, a peptide with the sequence of residues −18 to +10 of acarboxy-proprothrombin) increased NEM incorporation to 140 ± 12% (Table 1). A similar result was observed when FLAG-carboxylase was incubated with the synthetic substrate FLEEL either in the presence or in the absence of propeptide. In these reactions, NEM incorporation was increased to 170 ± 4% and 220 ± 0.19%, respectively (Table 1). These data demonstrate that the binding of a glutamic acid-containing substrate to the carboxylase results in increased accessibility of a free cysteine residue(s) to NEM.

We have previously observed that the binding of glutamic acid-containing substrate converts the vitamin K-dependent carboxylase from an inactive to an active epoxidase *(18)*. We hypothesized that the availability of a free cysteine residue(s) in the carboxylase for interaction with vitamin KH_2 might be regulated by occupancy of the glutamate substrate binding site. Thus, experiments were performed to assess the effect of vitamin KH_2 on NEM incorporation using conditions which result in complete incorporation of NEM into the carboxylase (5 mM NEM, 24 °C). Incubation of FLAG-carboxylase with vitamin KH_2 alone had no effect on NEM incorporation (100 ± 8%) (Table 2). However, when FLAG-carboxylase was incubated with vitamin KH_2 in the presence of propeptide plus glutamic acid-containing substrate, approximately half as much NEM was incorporated into the carboxylase compared to reactions performed in the absence of vitamin KH_2 (Table 2). Similar results were obtained whether proPT28 or proPT18 plus FLEEL was used as the glutamyl substrate (59 ± 0% and 49 ± 7%, respectively) (Table 2). This result appeared to be independent of the presence of propeptide since similar results were observed when vitamin KH_2 was incubated with FLEEL in the absence of propeptide (56 ± 2%) (Table 2). As expected, vitamin KH_2 had no effect on NEM incorporation in the presence of propeptide alone (97 ± 6%) (Table 2). These data indicate that vitamin KH_2 can compete with NEM for occupancy of a binding site which includes a free cysteine residue(s) in the carboxylase but only when the glutamate substrate binding site of the enzyme is occupied.

DISCUSSION

In the current study, the role of free cysteine residues in the enzymatic activity of the vitamin K-dependent carboxylase was examined. Previous studies using sulfhydryl-reactive reagents have suggested a role for free cysteine residues in the function of the vitamin K-dependent carboxylase; however, these studies were performed using crude bovine liver carboxylase preparations *(19−23)*, or carboxylase purified from bovine liver in the presence of propeptide *(24)*. The nature of the enzyme preparations previously used limited the scope of the analyses of the role of free cysteines that could be accomplished. The recombinant, FLAG epitope-tagged bovine vitamin K-dependent carboxylase used in the current study is free of both endogenous substrate and free propeptide, allowing us to study the influence of these species, as well as vitamin KH_2, on the inhibition of carboxylase by sulfhydryl reagents. Using recombinant, FLAG-carboxylase, we also observed that sulfhydryl-reactive reagents inhibited both the carboxylase activity and the epoxidase activity of the enzyme. This result was not unexpected since it has been demonstrated previously that the production of γ-carboxyglutamic acid and the production of vitamin K 2,3-epoxide are tightly coupled in both crude (*31*) and purified *(24)* carboxylase systems.

NEM forms an irreversible alkyl derivative with free sulfhydryl groups that is resistant even to acid hydrolysis (*32*). At relatively high concentrations of NEM and/or high pH, side reactions with NEM and the imidazole group of histidine or α-amino groups of amino acids may occur. Since side reactions were unlikely to occur under the conditions used in this study, our stoichiometric analyses indicate that NEM reacts with 2−3 cysteine residues in the carboxylase. Half-maximal incorporation of NEM into the carboxylase occurred slightly earlier in time than half-maximal inhibition of enzyme activity, suggesting that the cysteine residue(s) derivatized most rapidly may not be the one(s) important for catalysis.

The presence of either FLEEL or proPT28 both enhances the rate of incorporation of NEM into at least one free cysteine residue in the carboxylase and makes NEM derivatization susceptible to competition from vitamin KH_2. These results are consistent with our previous observation that glutamic acid-containing substrate activates vitamin K epoxidase activity *(18)*. We hypothesize that the binding of a glutamyl substrate to the carboxylase activates vitamin K epoxidation by inducing a conformational change in the

enzyme which makes a critical cysteine residue(s) accessible for interaction with vitamin KH_2. This critical cysteine residue is always accessible to NEM as the carboxylase can be inactivated by incubation with sulfhydryl-reactive reagents in the absence of glutamate containing carboxylase substrate. In addition, while NEM forms a covalent adduct with a cysteine residue, the interaction of vitamin KH_2 would be reversible. Finally, NEM derivatizes 2−3 cysteines in the carboxylase. It is thus noteworthy that in the presence of a glutamate substrate vitamin KH_2 can decrease NEM incorporation by 50%. This strongly supports the schema shown in Figure 1, which predicts the critical importance of a free cysteine residue in initiating vitamin K oxygenation.

In earlier studies, we showed that both proFIX18 (a peptide based on residues −18 to −1 of profactor IX) and FLEEL could activate the epoxidase activity of the carboxylase although the effect of FLEEL was greater than that of proFIX18. In the current studies, proPT18 alone neither enhanced incorporation of NEM into carboxylase nor facilitated inhibition of NEM incorporation by vitamin KH_2. All of our experience with this system indicates that these differences cannot be attributed to differences between proFIX18 and proPT18 (for example, see *33*). The current studies were done at lower concentrations of propeptide, and the enzymatic assay for epoxide formation may be more sensitive than the assay for NEM incorporation. Thus, lack of an effect of propeptide on NEM incorporation into the carboxylase may be a result of detection limits. Alternatively, propeptide may effect a step along the pathway to epoxide formation that is different from that effected by FLEEL and one that does not involve the critical modified cysteine residue. That this is the case may be suggested by comparison of the combined effect of both propeptide and FLEEL on epoxide formation versus NEM incorporation. In our earlier studies, we found that the presence of both proFIX18 and FLEEL was at least an order of magnitude more effective in stimulating epoxide formation than either proFIX18 or FLEEL alone. In contrast, the effect of proPT18 plus FLEEL on NEM incorporation into carboxylase is equivalent to that of FLEEL alone. This supports the suggestion that the enhancement of epoxide formation by propeptide is not related to exposure of a cysteine residue critical for interaction with vitamin KH_2 and that the effects of propeptide and FLEEL on the epoxidase activity of the carboxylase are additive.

In our studies, propeptide binding to the carboxylase has no effect on exposure of the cysteine residue involved in vitamin K epoxidation. Indeed, an exact role for the propeptide in vitamin K epoxidation by the carboxylase has not been demonstrated. Recent kinetic studies demonstrated that the propeptide enhancement of carboxylase catalytic efficiency is in selective lowering of an energy barrier preceding the γ-glutamyl carbanion intermediate either by accelerating formation of the reactive vitamin K intermediate or by proton abstraction from a substrate glutamyl residue *(34)*. The identities of the propeptide and glutamyl substrate binding sites in the carboxylase are unclear (for review, see *1*).

In an earlier study, incubation with vitamin KH_2 in the absence of added glutamyl substrate prevented inhibition of epoxidase activity, but not carboxylase activity, by NEM *(23)*. These experiments used an impure bovine liver carboxylase preparation that likely contained contaminating, endogenous glutamic acid-containing substrate. In our experiments using FLAG-carboxylase which is >90% pure and has not been exposed to propeptide or glutamic acid-containing substrate inhibition of NEM incorporation by vitamin KH_2 is dependent upon the binding of glutamic acid-containing substrate to the carboxylase. Based upon the observations of Canfield (*23*), Naganathan et al. proposed that the vitamin K-dependent carboxylase contains two active site thiols, one of which is protected from NEM inactivation by vitamin K hydroquinone *(25)*. In this scheme, one of the sulfhydryl groups abstracts a proton from vitamin K hydroquinone to initiate its oxygenation; the other serves to hydrogen bond CO_2 at the active site. Although stoichiometric analyses indicate that NEM binds to 2−3 cysteine residues, we have no direct evidence that more than one cysteine residue is important for catalytic activity. However, we observe that the initial rate of NEM inhibition of carboxylation is faster than the initial rate of NEM inhibition of vitamin K epoxidation, which is consistent with the notion that the carboxylase active sites contain more than one thiol group. If only one thiol group was catalytically important, then the initial rate of NEM inhibition of carboxylation would be the same as the initial rate of NEM inhibition of vitamin K epoxidation since γ-carboxylation is absolutely dependent upon vitamin K epoxide formation. Thus, our data are consistent with but cannot prove the participation of two free thiol groups in the carboxylase as proposed by Canfield *(23)* and by Naganathan *(25)*.

At this time, the identity of the cysteine residues which play a role in the epoxidation of vitamin K is unknown. The cDNAs for bovine *(35)*, human *(36)*, and rat *(37)* carboxylase have been cloned. The bovine isoform has 11 cysteine residues; the human isoform has 10; and the rat isoform has 9. Eight cysteine residues are conserved among the three species. The cysteine residue that is important for catalysis will most likely be found among these eight cysteine residues. Recently, Bala Nirmala and Berkner have mutated cysteine 343 to serine in bovine carboxylase *(38)*. Their data show that this mutation abolishes the carboxylase and epoxidase activities of recombinant vitamin K-dependent carboxylase produced in insect cells. It should be noted that cysteine 343 is one of the eight cysteine residues that is conserved among the bovine, human, and rat isoforms of the carboxylase. However, their data do not distinguish between a role for this cysteine residue in activity of the enzyme or in maintaining structural integrity of the enzyme. Future studies will be directed at the unequivocal identification of the important cysteine residue(s).

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