BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **251,** 22–26 (1998)
ARTICLE NO. RC989410

Identification of the Five Hydrophilic Residues (Lys-217, Lys-218, Arg-359, His-360, and Arg-513) Essential for the Structure and Activity of Vitamin K-Dependent Carboxylase

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Received August 31, 1998

Vitamin K-dependent carboxylase catalyzes the posttranslational conversion of glutamic acid to γ-carboxyglutamic acid in vitamin K-dependent proteins. The clustered charged-to-alanine scanning mutagenesis of bovine carboxylase has identified five distinct candidate regions (I. Sugiura *et al., J. Biol. Chem.* 271, 17837–17844, 1996) with significant loss-of-function phenotype. To further specify the residues essential for the structure and function of the enzyme, Lys-217, Lys-218, Arg-359, His-360, Lys-361, Arg-513, and Lys-515 were analyzed by substituting to alanine individually. All the mutants except for K217A were expressed in Chinese hamster ovary cells. The carboxylase activities of R359A, H360A, and R513A decreased in parallel with the vitamin K epoxidase activities. Both carboxylations by R359A and H360A were stimulated saturatively at 1 μM factor IX propeptide (pro-FIX18) concentration, but that by R513A was not at a concentration up to 128 μM. K218A completely lost the enzyme activities but it cross-linked to the propeptide, suggesting that Lys-218 is critical for enzyme activity without affecting propeptide binding. We conclude that Lys-218, Arg-359, and His-360 are involved in the catalytic event, and Arg-513 participates in propeptide binding.

The vitamin K-dependent γ-glutamyl carboxylase (carboxylase) catalyzes the post-translational modification of 9-12 residues of glutamic acid to γ-carboxylglutamic acid in the vitamin K-dependent proteins, including blood coagulation factors (prothrombin, factor VII, factor IX, factor X, protein C, and protein S) and bone proteins (osteocalcin or matrix Gla protein). The Gla residues are essential for maintaining Ca^{2+}-dependent conformation which enables the proteins to bind to phospholipid surfaces of the cells. A propeptide region preceding the Gla domain in the precursor forms of the vitamin K-dependent proteins serves as a docking element called γ-carboxylation recognition site (γ-CRS) (1).

The γ-carboxylation is a complex chain of chemical reactions which require oxygen, CO_2, and vitamin K hydroquinone (vitamin KH_2). Vitamin KH_2 is converted to vitamin K 2,3 epoxide by the epoxidase activity of carboxylase and a very strong base generated during the oxidation step removes a proton from the γ-carbon of glutamic acid (2). As the result, the glutamate carbanion undergoes carboxylation to yield γ-carboxyglutamic acid.

Amino acid sequences deduced from the cDNA encoding bovine or human carboxylases indicate a single-chain 94-kDa polypeptide that has potential transmembrane regions within N-terminal half of the protein (3). A hydrophilic C-terminal half of the protein has potential N-linked glycosylation sites.

Several studies have been performed to localize functional regions of the carboxylase (4–8). Kuliopulos *et al.* employed N-bromoacetyl-peptide substrate affinity labeling and suggested that substrate binding site is within the first 218 residues (4). Results of deletion mutagenesis suggested that binding site for vitamin KH_2 is located within the region between residues 676 and 711 (5). For the localization of propeptide binding site, a cross-linking study of labeled factor IX propeptide have identified the propeptide binding site to the region between residues 50 and 225 (6), but another study to the C-terminal region beyond residue 438 (7).

Site-directed mutagenesis of the total 41 charged amino acid residues of bovine carboxylase have identified 5 clustered regions essential for the structure and/or the function of the enzyme (8). Triple mutations at Arg-359, His-360, and Lys-361 (R359A/H360A/

0006-291X/98 $25.00

K361A) resulted in impaired catalytic activities but a double mutant K217A/K218A showed complete loss of activities. On the other hand, R513A/K515A showed the significant decrease in the binding to the propeptide (8). In the present study, we analyze each single residue to convert to alanine individually in the clustered mutants K217A/K218A, R359A/H360A/K361A, and R513A/K515A. The analysis specifies functional roles of each amino acid residue for either the enzyme activities or the propeptide binding, thus identifying the mechanism of action of carboxylase.

MATERIALS AND METHODS

Materials. Polymerases and restriction endonucleases were purchased from New England Biolabs (Beverly, MA). The expression vector pED and CHO-Dukx-B11 were gifted from Genetics Institute. Lipofectin and other cell culture reagents were purchased from Life Technologies (Gaithersburg, MD). ECL-Western blotting detection reagents, Hyperfilm-ECL, NaH[^{14}C]O3, Na[^{125}I], and peroxidase-conjugated goat anti-mouse immunoglobulin were purchased from Amersham (Buckinghamshire, England). Anti-FLAG M2 monoclonal antibody and FLAG-bacterial alkaline phosphatase were obtained from Eastman Kodak (Rochester, NY). Vitamin K1 (10 mg/ml) obtained from Eisai corporation (Tokyo, Japan) was chemically reduced with 8 mg of $NaBH_4$. Phe-Leu-Glu-Glu-Tyr (FLEEL), L-α phosphatidylcholine (PC) and 3-[(3-cholamindopropyl) dimethylammonio]-1-propanesulfonic acid (CHAPS) were from Sigma (St. Louis, MO). Iodobeads and chemical cross-linker, DSS were purchased from Pierce (Rockford, IL). Peptides proFIX18 and proFIX19 were provided by B. C. Furie (New England Medical Center, MA). C18 Sep-Pak column were purchased from Millipore (Milford, MA). DC Protein Assay was obtained from Bio Rad (Hercules, CA).

Transfection, cell culture, and cell line selection. CHO-Dukx-B11 (9) was used to express the carboxylase species as described elsewhere (8). Cells were transfected with pED/FLAG-CBX containing either the wild type or mutant carboxylase cDNA fused to FLAG -Tag by the Lipofectin method according to the manufacture's recommendation. Cells from selected colonies were grown in vitamin K-containing α-MEM/FCS.

Preparation of cell lysate. Fully confluent cells in T500 flasks were resuspended in Phosphate buffered saline, pH 7.4 (PBS), 5 mM EDTA, washed once with 10 ml of PBS, and then resuspended in PBS, 20% glycerol, and protease inhibitor cocktail (PIC; 2 mM dithiothreitol, 2 mM EDTA, 0.5 mg/ml leupeptin, 1 mg/ml pepstatin A, 2 mg/ml aprotinin). One milliliter of cell suspension was mixed with 1 ml of PBS, 1% CHAPS, 0.2% PC, 20% glycerol, and PIC. Suspended cells were sonicated three times for 5 seconds. After centrifugation (16,000g, 10 min, 4°C), 50 μl aliquot of the solubilized supernatant was assayed by DC protein assay to determine the protein concentration. All these procedures were strictly performed on ice and samples were immediately stored at −80°C before use. Quantity analysis of recombinant wild-type and mutant FLAG-CBX were determined by Western blotting analysis using anti-FLAG M2 monoclonal antibody and known concentrations of FLAG-bacterial alkaline phosphatase as standard (8).

Assay of carboxylase and epoxidase activity. Detailed assay procedures were according to those by Sugiura *et al.* (8). The carboxylation activity in the cell lysate was studied by quantitating the amount of [^{14}C]O_2 incorporated into peptide substrates FLEEL. For a mutant R513A, 56 μM of vitamin K was used instead of 222 μM (10). Vitamin K epoxide formation was determined by calculating the amount of vitamin K epoxide (5) separated by reverse-phase C18 HPLC column (TOSOH, Tokyo, Japan). Results were adjusted according to the total protein amounts of each cell lysate after subtracting the activities of untransfected CHO cells.

TABLE 1

Specific Activities of Mutant Carboxylases[a]

	Specific carboxylase activity (pmol/min)	Specific epoxidase activity (pmol/min)	Ratio of specific activities of carboxylation to epoxidation
Wild type	22.3 ± 1.2	47.5 ± 9.6	0.47
K218A	ND	ND	ND
R359A	16.2 ± 0.5	30.8 ± 7.2	0.53
H360A	14.5 ± 0.8	28.9 ± 8.6	0.50
K361A	24.2 ± 0.3	59.2 ± 30.1	0.41
R513A	6.5 ± 0.1	15.9 ± 1.4	0.41
K515A	24.0 ± 0.3	37.8 ± 26.4	0.63

[a] A mutant K217A was not expressed. Values indicate means ± SD of two independent duplicated experiments. ND, not detected.

Purification of wild type carboxylase and mutant K218A. Purification method of FLAG-tagged recombinant carboxylase was described elsewhere (11). Briefly, large-scale preparation of cells from stable colonies producing wild-type and mutant K218A were grown in each 6600-cm^2 tissue culture flasks (Cell Factory, NUNC) up to confluence. Cell lysates were prepared as above, centrifuged at 150,000g for 1 h at 4°C, and the supernatant was applied to affinity chromatography (Anti-FLAG M2 monoclonal antibody affinity resin). Bound enzyme was eluted by PBS/20% glycerol/0.5% CHAPS/0.2% PC containing varying amount of FLAG-peptide at 5, 10, 20, 50, 75, 100, 150, 200, and 400 μg/ml. The isolated enzyme was stored at −80°C.

Chemical cross-linking study. Peptide proFIX19 was radiolabeled with Na[^{125}I] using Iodobeads and free Na[^{125}I] was separated from the peptide by C18 Sep-Pak chromatography. Fractions containing [^{125}I]-proFIX19 were air-dried and stored at 4°C. ProFIX19 was used instead of proFIX18 because proFIX18 has no tyrosine residue required for iodine labeling. Cell lysate solutions (200 μl) of wild type and K218A from 1×10^8 cells were centrifuged at 150,000g, 4°C for 1 h and the supernatant was used for the cross-linking reaction. The reaction procedure was described by Wu *et al.* (7) and the samples were analyzed by non-reduced SDS–PAGE followed by autoradiography using BAS 2000 system (Fuji, Tokyo, Japan). The gel was subsequently subjected to the Western blotting analysis as described above.

RESULTS

Expression of carboxylase mutants. In the present study, we produced 7 single alanine mutants of bovine carboxylase. The amount of expressed recombinant proteins in the CHO cell lysate was analyzed by Western blotting. K217A was not expressed at all but other mutants were expressed with normal levels (data not shown). Thus, six mutant enzymes were studied (Table 1). Wild type and mutant enzymes had similar molecular weight, but K218A had slightly faster electrophoretic mobility, as observed in case of a double-mutant K217A/K218A (8).

Carboxylation and epoxidation activity of recombinant carboxylases in CHO cell lysate. Specific carboxylase and epoxidase activities of recombinant enzymes

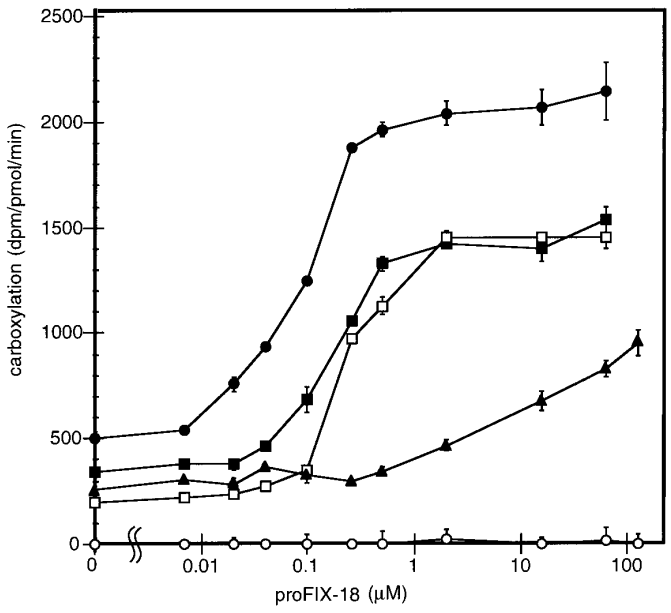

FIG. 1. Effect of proFIX18 on carboxylation. The effect of increasing concentration of proFIX18 on the carboxylation was studied for the wild type and four mutant carboxylases. The carboxylation activity was expressed as the amount of $[^{14}C]O_2$ incorporated into 1 pmol of peptide substrates FLEEL. Closed circles, wild type carboxylase; closed squares, R359A; open squares, H360A; closed triangles, R513A; open circles, K218A. Values indicate the averages of two distinct duplicate experiments.

in the cell lysates were quantitated in the presence of 16 μM proFIX18 and 222 μM vitamin KH_2. Both specific activities were adjusted by dividing with the total protein amounts of each cell lysate specimen after subtracting the activities of untransfected CHO cells. Results of six mutants as well as wild type are summarized in Table 1. Two mutants, K361A and K515A, had normal levels of both carboxylase and epoxidase activities. In contrast, four mutants, K218A, R359A, H360A, and R513A, exhibited significantly decreased carboxylase activities in parallel with the epoxidase activities (Table 1), suggesting that these four residues are involved in the catalytic process of bovine carboxylase.

Stimulation of carboxylation activity by proFIX18. Carboxylation and epoxidation activities are known to be regulated by the propeptide of vitamin K-dependent coagulation proteins. To compare the stimulatory effects of the propeptide on the activity of mutant enzymes, we studied the carboxylation in the presence of varying concentrations of the factor IX propeptide, proFIX18. Experiments were performed for the mutants K218A, R359A, H360A, and R513A of which the cell lysate exhibited the decrease in the enzyme activities (Table 1). Figure 1 demonstrates the effect of the increasing concentration of proFIX18 on the carboxylation activity of wild-type carboxylase. The activity of R359A and H360A showed similar dependence on proFIX18 concentration as of wild type, although the peak ^{14}C incorporation at the maximum proFIX18 concentration was approximately 60% of a wild-type carboxylase. This suggests that R359A and H360A have normal affinities for the factor IX propeptide. R513A, however, showed the delayed dependence on the increasing proFIX18 concentration. While up to 128 μM of the propeptide concentration was tested, the peak ^{14}C incorporation could not been reached to a plateau level, suggesting that R513A apparently has decreased affinity for proFIX18 but is sensitive to its stimulation. On the other hands, K218A had no carboxylation and epoxidation activity (Table 1) and increased concentration of proFIX18 failed to stimulate K218A for the carboxylation activity (Fig. 1), indicating that the mutation at K218 resulted in the complete loss of carboxylation function.

Chemical cross-linking study. We then studied whether or not the mutation at K218 disrupts the binding to propeptide. In the presence of chemical cross-linker DSS, CHO cell lysates containing recombinant wild type or K218A, were incubated with ^{125}I-labeled proFIX19 and analyzed by non-reducing SDS–PAGE (Fig. 2A). ^{125}I-proFIX19 bound to both wild type and K218A showing approximately 101-kDa bands (lanes 2, 3, and 4 in Fig. 2A). The 101-kDa band was not detected in mock control (lane 5). The gel was electroblotted on PVDF membranes followed by the incubation with anti-FLAG M2 monoclonal antibody (Fig. 2B). Anti-FLAG M2 antibody reacted with the 101-kDa cross-linking band in both wild type and

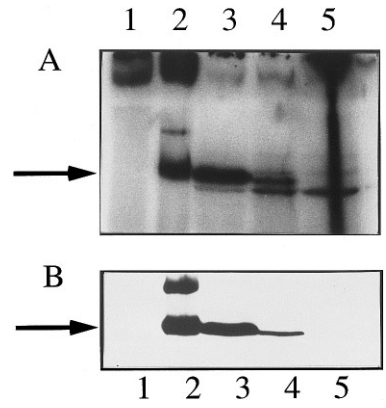

FIG. 2. Chemical cross-linking study. Using chemical cross-linker DSS, recombinant wild type or K218A were cross-linked with ^{125}I-labeled proFIX19 and analyzed by SDS–PAGE and Western blotting. ^{125}I-proFIX19 alone was also subjected to the cross-linking reaction. (A) Lane 1, ^{125}I-proFIX19 alone; lane 2, purified wild type (as shown in lane 2 of Fig. 3); lane 3, cell lysates of wild type; lane 4, cell lysate of K218A; lane 5, mock control. The approximately 101-kDa band in size that corresponds to cross-linked carboxylase are shown by the arrow. (B) The same gel was electroblotted on the PVDF membrane followed by incubation with anti-FLAG M2 monoclonal antibody. The 101-kDa bands are shown by the arrow.

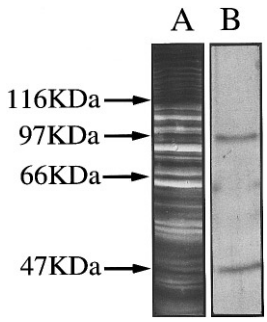

FIG. 3. SDS–PAGE analysis of purified K218A. Reduced protein samples were electrophoresed on the 10% SDS-polyacrylamide gel and visualized with silver staining. The positions of migration of molecular markers are indicated on the left. Lane A, cell lysate expressing K218A. Lane B, purified K218A. A 97-kDa band indicates the mutant carboxylase (11).

K218A, but not in mock control, indicating that the ^{125}I-proFIX19 bound to both wild type and K218A.

Carboxylation and epoxidation by purified recombinant enzyme. We then evaluated the ability of purified K218A to catalyze varying concentrations of FLEEL or vitamin KH_2. Recombinant wild type carboxylase and K218A were purified by using anti-FLAG M2 affinity chromatography. In Fig. 3, a 97-kDa band indicates the mutant carboxylase, whereas the origin of a 45-kDa band is not known. However, the presence of the 45-kDa band is known not to affect the activity of carboxylase (11).

We studied the carboxylation and epoxidation activities of purified K218A. K218A showed no carboxylation activity although the maximum concentration of the substrate FLEEL and vitamin KH_2 were used (data not shown). Then, the propeptide dependency of epoxidation in the absence of FLEEL was studied for purified wild type and K218A enzymes. While vitamin K epoxide formation by a wild type enzyme depended on the proFIX18 concentration and was saturated at 1 μM propeptide, K218A showed no detectable epoxidation (Fig. 4).

DISCUSSION

A previous site-directed mutagenesis study indicated that a double mutant R513A/K515A bound to the propeptide with lower affinity than of wild type carboxylase (8). The study also demonstrated that R359A/H360A/K361A resulted in normal affinity for propeptide, but exhibited a reduced catalytic rate for carboxylation. Mutations at K217A/K218A was shown to completely abolish the activities (8). In the present paper, we studied each single amino acid residue in these clustered mutants to determine the requirement of amino acid residues essential for the carboxylase activity.

Mutant as well as wild type carboxylases were FLAG-tagged and expressed in the CHO cells. All the mutants except for K217A were expressed within similar levels compared to that of wild type carboxylase, being consistent with the hypothesis that K217 is important for protein conformation or secretion.

Mutants K361A and K515A had normal activities. Four mutants, K218A, R359A, H360A, and R513A, exhibited decrease in the specific enzyme activities and it was in parallel both in the specific carboxylation and epoxidation activities (Table 1). These facts indicate that the impaired activity of clustered mutant R359A/H360A/K361A is represented by residues Arg-359 and His-360, and that of clustered K217A/K218A is by Lys-218.

When varying concentrations of proFIX18 were used, carboxylation activity of R359A and H360A was dependent on the propeptide concentrations as seen in the wild type enzyme (Fig. 1), suggesting that R359A and H360A appear to have normal propeptide affinity. Therefore disruptions at R359 and R360 may impair the catalytic function of carboxylase without affecting the propeptide binding.

We observed that the maximum concentration of proFIX18 (128 μM) was not sufficient for the stimulation of carboxylation activity of mutant R513A (Fig. 1). It is thus possible that R513A binds to propeptide with lower affinity than wild type, R359A or H360A. Recently, two distinct amino acid segments have been proposed as potential propeptide binding sites (6, 7). Yamada *et al.* used a peptide proFIX19 of which a Phe residue is replaced by the photoactive cross-linking

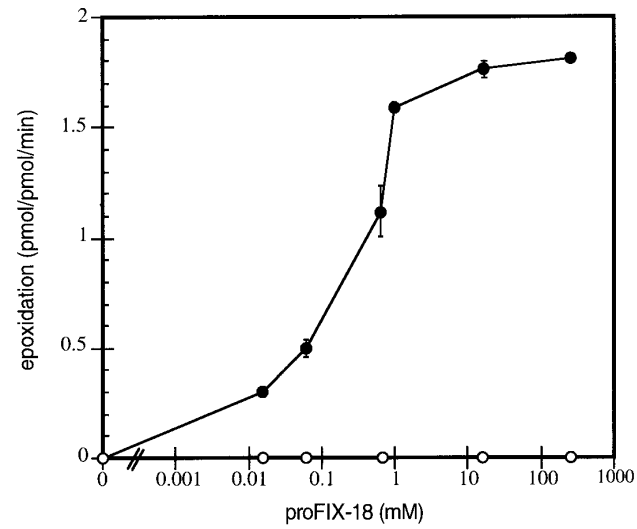

FIG. 4. Effect of proFIX18 on epoxidation in the absence of FLEEL. Epoxidation of 222 μM vitamin KH_2 in the presence of various concentrations of proFIX18 by the purified wild-type carboxylase (closed circles) or K218A (open circles). The epoxidation activity was determined calculating the amount of vitamin K epoxide separated by reverse-phase HPLC (5).

benzoylphenilalanine, and demonstrated its covalent cross-linking to the region between residues 184 and 225 of bovine carboxylase (6). By using chemical cross-linker DSS, however, Wu *et al.* demonstrated that ^{125}I-labeled proFIX19 bound to the region between residues 438 and 579 of bovine carboxylase (7). Our mutant R513A is included in the region defined by the latter case, interpreting that R513 may contribute to the propeptide binding. Nonetheless, further studies will be required to identify the critical propeptide binding site(s) of carboxylase.

Mutant K218A had undetectable activities of both epoxidation and carboxylation, and carboxylation by K218A was not detected by the maximum concentration (128 μM) of the propeptide (Fig. 1). However, the cross-linking study demonstrated that K218A associated with the propeptide normally, suggesting that K218 is not involved in the propeptide binding (Fig. 3).

We then purified K218A to examine whether it has completely lost the activities, or persevere an activity which is detectable in the purified system (10). First, purified K218A was analyzed for the ability of carboxylation at varying concentrations of proFIX18, vitamin KH_2, and FLEEL but K218A did not respond to any stimulatory effects (data not shown). We then tested, in the absence of FLEEL, the vitamin KH_2 epoxidation in the presence of varying concentrations of the propeptide. While the epoxidation by wild type carboxylase responded to the increasing concentrations of the propeptide, K218 showed no epoxidation activity (Fig. 4). These findings indicate that K218 is critically required for catalytic functions of carboxylase, without affecting the propeptide binding.

However, it is not clear how Lys-218 acts during the entire catalytic steps of carboxylase. By Dowd *et al.*, the free energy of oxygenation of vitamin K hydroquinone (vitamin KH_2) is used to transform a weak base to a strong base in order to promote proton removal from selected glutamate (Glu) residues in the blood clotting proteins (12). Moreover, vitamin K epoxidation by the purified carboxylase could be detected in the absence of FLEEL (10). In this context, the epoxidation reaction independently could precede the carboxylation reaction. But using the same purified system as that of Sugiura *et al.* (10), we could not detect the epoxidase activity of K218A, raising the possibility that the loss of carboxylation activity of K218A might be due to the loss of primary epoxidation activity. Further studies, however, will be required to verify the distinctive role(s) of K218 in the course of modification of Glu residues of the blood clotting proteins.

In conclusion, Arg-359, His-360, and Lys-218 are involved in catalytic event with normal propeptide binding capacity. Arg-513 appears to be involved in the propeptide binding.

ACKNOWLEDGMENTS

We thank Dr. Bruce Furie and Barbara C. Furie (Tufts University School of Medicine, Boston, MA) for providing proFIX18 and proFIX19 and Chika Wakamatu, Mieko Suzuki, and Satoshi Suzuki for their technical help.

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