The Propeptide Binding Site of the Bovine γ-Glutamyl Carboxylase*

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γ-Glutamyl carboxylase is an integral membrane protein required for the posttranslational modification of vitamin K-dependent proteins. The main recognition between the enzyme and its substrates is through an 18-amino acid propeptide. It has been reported that this binding site resides in the amino-terminal third of the γ-glutamyl carboxylase molecule (Yamada, M., Kuliopulos, A., Nelson, N. P., Roth, D. A., Furie, B., Furie, B. C., and Walsh, C. T. (1995) Biochemistry 34, 481–489). In contrast, we found the binding site in the carboxyl half of the γ-glutamyl carboxylase. We show that the carboxylase may be cleaved by trypsin into an amino-terminal 30-kDa and a carboxyl-terminal 60-kDa fragment joined by a disulfide bond(s), and the propeptide binds to the 60-kDa fragment. The sequence of the amino terminus of the 60-kDa fragment reveals that the primary trypsin-sensitive sites are at residues 349 and 351. Furthermore, the tryptic fragment that cross-links to the propeptide also reacts with an antibody specific to the carboxyl portion of the γ-glutamyl carboxylase. In addition, cyanogen bromide cleavage of bovine γ-glutamyl carboxylase cross-linked to the peptide comprising residues TVFLDHENANKILNRPKRKY of human factor IX yields a cross-linked fragment of 16 kDa from the carboxyl half of the molecule, the amino-terminal sequence of which begins at residue 438. Thus, the propeptide binding site lies carboxyl-terminal to residue 438 and is predicted to be in the lumen of the endoplasmic reticulum.

γ-Glutamyl carboxylation, accomplished by the enzyme γ-glutamyl carboxylase, is a postranslational modification essential for the biological activities of a number of vitamin K-dependent proteins. The importance of γ-glutamyl carboxylation is demonstrated by the various functions of the vitamin K-dependent proteins. Not only does the propeptide of the various vitamin K-dependent proteins function as a recognition site for the enzyme γ-glutamyl carboxylase. This hypothesis was confirmed by Knobloch and Suttie (9), who demonstrated that the factor X propeptide stimulated the incorporation of 14CO2 into the substrate FLEEL. The recognition site was further elucidated by Jorgensen et al. (10). Subsequently, propeptide-containing peptides were demonstrated to have a Km that was three orders of magnitude lower than the pentapeptide substrate FLEEL for the enzyme γ-glutamyl carboxylase (11, 12).

Further elucidation of the mechanisms of action of the carboxylase was hindered by the lack of a purified enzyme. To begin a systematic study we made a recombinant peptide, FIXQ/S, which contains the propeptide and the complete Gla domain sequence of human factor IX (12), and successfully used it as an affinity ligand to purify γ-glutamyl carboxylase to near homogeneity in a single step purification (13). We were then able to clone the complete cDNA sequence of γ-glutamyl carboxylase using the deduced amino acid sequence of a tryptic fragment (14).

The interaction between γ-glutamyl carboxylase and its substrate is one of the most interesting aspects of γ-glutamyl carboxylation, and the major factor determining this interaction is the propeptide of the various vitamin K-dependent proteins. Not only does the γ-glutamyl carboxylase recognize the propeptide sequence, but it also processes multiple modification sites in a single substrate molecule by a progressive mechanism (15).

We report here that the major propeptide binding site is in the hydrophilic, soybean seed lipoxygenase-like domain of the carboxylase molecule, which is predicted to reside in the lumen of the endoplasmic reticulum. This is in contradiction to the site proposed by Yamada et al. (16) and Sugiuara et al. (17).

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1 The abbreviations used are: FIXQ/S, recombinant peptide comprising residues 18 to +41 of profactor IX with mutations T18A, R4Q, R1S, and M19R; pro-FIX9, peptide comprising residues TVFLDHENANKILNRPKRKY of human factor IX; pro-FIX19, peptide comprising residues TVFLDHENANKILNRPKRKY; BPA, p-benzoyleglycolalanine; DSS, disuccinimidyl suberate; PDDV, polydiyldiene difluoride; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate; Endo H, endoglycosidase H; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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EXPERIMENTAL PROCEDURES

Materials—All chemicals were reagent grade. Disuccinimidyl suberate (DSS) and Iodobeads were purchased from Pierce. NaCl was from DuPont NEN. CentriPrep-30 was from Amicon. Immobilon-P was from Millipore. PVDF membranes were purchased from Bio-Rad. The ECL Western blotting detection reagent was from Amersham Corp. SP-Sepharose was from Pharmacia Biotech Inc. Protease inhibitors H-o-Phe-Phe-Arg-chloromethylketone and H-o-Phe-Pro-Arg chloromethylketone were from Bachem. Aprotinin, pepstatin A, and trypsin were purchased from Boehringer Mannheim. Leupeptin, phenylmethylsulfonyl fluoride, and CHAPS were obtained from Sigma. Peptides pro-FIX19 (AVFGLDHEANKILNRPKRY), pro-FIX19-16BPA (TVFGLDHEANKILNRPKRY), pro-FIX19-16BPA (TVFGLDHEANKILNRPKRY), B is p-benzoylphenylalanine (BPA), hbcG220–234 (CDADWVEGYSMEYLS), and hbcG709–723 (GGRPSLEQLAQEVTYA) were obtained from Chiron (an amino-terminal cysteine was added to hbcG220–234 and hbcG709–723 for conjugation to keyhole limpet hemocyanin). Recombinant peptide FIXQ/S was prepared as described (12).

Cleavage of γ-Glutamyl Carboxylase by Limited Trypsinization—γ-Glutamyl carboxylase was affinity-purified by elution method II as described (13). pro-FIX19 and protease inhibitors (when used) were removed before trypsin cleavage by ultrafiltration using a CentriPrep-30 unit with deCIP buffer (25 mM MOPS, pH 7.5, 500 mM NaCl, and 0.3% CHAPS). A pilot reaction was run for each batch of γ-glutamyl carboxylase and trypsin to determine the optimal condition for limited trypsin cleavage. Because limited trypsin cleavage could only be obtained with intact, enzymatically active γ-glutamyl carboxylase, the cleavage was carried out at 4 °C. Trypsin cleavage was stopped by adding H-o-Phe-Pro-Arg-chloromethylketone to a final concentration of 1.6 μM.

DSS-mediated Chemical Cross-linking—Peptides pro-FIX19 and FIXQ/S were radiolabeled with Na125I using Iodobeads according to the manufacturer. To increase cross-linking efficiency, the enzymatically active, intact or the limited trypsinized γ-glutamyl carboxylase (in elution buffer) was preincubated with propeptide-containing peptides (e.g., 12 μg 125I-peptide or 0.5–2 μg 125I-peptide with 40–100-fold excess unlabeled competitor) at 4 °C for 1 h before DSS was added to a final concentration of 50–200 μM. Cross-linking was carried out at 4 °C and at times varying from 20 min to 3 h before it was stopped with 50 mM Tris-HCl, pH 8.8.

Cross-linking pro-FIX19-16BPA to the γ-Glutamyl Carboxylase—pro-FIX19-16BPA (16) was cross-linked to the carboxylase by exposure to a 365-nm UV lamp (Cole-Parmer E-97600) for 15 min at a distance of 15 cm. During photo cross-linking the sample was maintained on ice.

Generation of Antibodies against Synthetic Peptides Derived from γ-Glutamyl Carboxylase Sequence—Synthetic peptides hbcG220–234 and hbcG709–723 were conjugated to keyhole limpet hemocyanin through an amino-terminal cysteine. One μg of conjugated peptide in Freund’s complete adjuvant was subcutaneously inoculated into New Zealand White rabbits. One microgram of each conjugated peptide in Freund’s incomplete adjuvant was used to boost the immunity 2 weeks after the first inoculation.

Identification of Tryptic Fragments by Immunoblotting—Forty μg of the limited trypsinized carboxylase was analyzed by reducing SDS-PAGE (18) and electroblotted onto Immobilon-P (Millipore). The membrane was probed with 200-fold diluted anti-hbcGC-amino acids antibody. ECL Western blotting detection reagent was used as recommended by the manufacturer to reveal the reactive fragment.

Determination of the Peptide Binding Region by Amino Acid Sequencing—γ-Glutamyl carboxylase was purified as described (13), except that 125I-pro-FIX19 or FIXQ/S was used in the elution. Ten to 100 μg of affinity-purified γ-glutamyl carboxylase was concentrated 6–8 fold on CentriPrep-30 units. Cross-linking reactions were carried out at 4 °C in 10–15 μl of 25 mM MOPS, pH 7.5, 500 mM NaCl, 15% glycerol, 0.4% phosphatidylcholine, and 0.25% CHAPS with 50–200 μM DSS. Sixty-five percent ammonium sulfate was used to precipitate the proteins from the pro-FIX19 cross-linking mixture. Proteins were dissolved in 5 μl of 25 mM MOPS, pH 6.5, 0.5% CHAPS, and 10% glycerol and sonicated for 10 s before being batch-adsorbed onto SP-Sepharose. The γ-glutamyl carboxylase and the cross-linked γ-glutamyl carboxylase were eluted from an SP-Sepharose column with a 0–1 M NaCl gradient in 25 mM MOPS, pH 7.5, 10% glycerol, 0.02% phosphatidylcholine, and 0.02% CHAPS. The γ-glutamyl carboxylase-containing fractions were further concentrated on CentriPrep-30, subjected to SDS-PAGE, electroblotted onto a nitrocellulose membrane, cleaved with cyanogen bromide, concentrated, refractionated by SDS-PAGE, and then analyzed by amino acid sequencing.

Sequence of the 30- and 60-kDa Fragments—The determination of the amino termini of the limited tryptic fragments was done at the Harvard Microchemistry Facility.

Quantitation of Radioactive Bands in PAGE—The radioactive bands were imaged with a Molecular Dynamics Storm 840 PhosphorImager, and quantitation was achieved with ImageQuant software.

RESULTS AND DISCUSSION

The γ-glutamyl carboxylase is a 758-amino acid protein that migrates on reducing SDS-PAGE as a 94-kDa protein. The enzymatically active γ-glutamyl carboxylase is cleaved by limited trypsin digestion into two major fragments with electrophoretic mobilities of approximately 60 and 30 kDa (Fig. 1, lane 3). The 60-kDa fragment reacts with an antibody made to a 15-amino acid peptide corresponding to residues 709–723, whereas the 30-kDa fragment reacts with an antibody made to residues 220–234 near the amino terminus of the γ-glutamyl carboxylase (Fig. 2). In the absence of the propeptide or during prolonged trypsin cleavage, the 60-kDa fragment is converted into a 50-kDa fragment. Because the 50-kDa fragment, which is identified with the cross-linked fragment, failed to react with an antibody made to residues near the carboxyl terminus of the 60-kDa fragment (Fig. 2), we postulated that the 50-kDa fragment arises by cleavage near the carboxyl terminus of the 60-kDa fragment. From the results of the immunoblot, we predicted that both the 60- and 50-kDa fragments were derived from the carboxyl half and the 30-kDa fragment from the amino-terminal half of the γ-glutamyl carboxylase. We also predicted that, although the 50- and 60-kDa fragments have different carboxyl-terminal sequences, they would have the same amino-terminal sequence.

To identify the primary trypsin-sensitive sites in the carboxylase, affinity-purified bovine γ-glutamyl carboxylase was cleaved by limited trypsin digestion, fractionated by reducing SDS-PAGE, and transferred to PVDF membranes for amino acid sequence analysis. Sequence analysis revealed that the 50- and 60-kDa fragments arise from cleavage following arginines 349 and 351 (Fig. 3). We also attempted to determine the sequence of the 30-kDa fragment. Analysis of amino acid composition predicted 15 pmol of the 30-kDa fragment compared with 7 pmol of the 60- and 50-kDa fragments in the sample that was used for sequence analysis. This result indicated that the blotting efficiency for each fragment was similar and predicts that, at each step in amino acid sequence analysis, the sum of the amino acid yield of the 50- and 60-kDa frag-
FIG. 2. Identification of the tryptic fragments of γ-glutamyl carboxylase by region-specific antibodies. A, reducing SDS-PAGE of γ-glutamyl carboxylase visualized by an antibody to residues 709–724 of the γ-glutamyl carboxylase. Lane 1, γ-glutamyl carboxylase; lane 2, its limited trypsin digestion product. B, as in A, except visualization is with an antibody made to residues 220–234 of the γ-glutamyl carboxylase. C, reducing SDS-PAGE of γ-glutamyl carboxylase visualized by silver staining. Lane 1, uncleaved γ-glutamyl carboxylase; lane 2, its limited trypsin digestion product.

FIG. 3. Sequence of the bovine γ-glutamyl carboxylase, * residues that differ from the human γ-glutamyl carboxylase. Boldface residues, those mutated in the study of Sugiura et al. 3

FIG. 4. Demonstration that the 30- and 60-kDa fragments of the γ-glutamyl carboxylase are joined by a disulfide bond(s). Lane 1, size markers; lane 2, trypsin-treated carboxylase subjected to SDS-PAGE; lane 4, same sample subjected to reducing PAGE. The samples are separated by a blank lane to prevent the reducing agent from diffusing between wells.

FIG. 5. Efficiency of DSS cross-linking. Silver-stained PAGE of γ-glutamyl carboxylase cross-linked to FIXQ/S.
or was DSS-mediated. Fig. 7 demonstrates that pro-FIX19-16BPA, with sequence identical to that used by Yamada et al. (16) was obtained and used for photochemical cross-linking. Fig. 7 shows that the cross-linking was accomplished photochemically and photochemically cross-linked it to iodinated pro-FIX19-16BPA. We then subjected the product to reducing and nonreducing SDS-PAGE and quantitated the amount of radioactivity in each band with a PhosphorImager. Fig. 8 shows a radioactive image of photochemically cross-linked γ-glutamyl carboxylase fractionated by nonreducing and reducing PAGE. The 60-kDa band of the reduced sample contained 87% of the radioactivity found in the 94-kDa unreduced γ-glutamyl carboxylase. Approximately 5% appeared in the 30-kDa band, and the remainder was in the uncleaved 94-kDa band. Thus, there was no selective loss of a cleaved fragment in our preparation.

We have made numerous attempts to narrow further the region to which the propeptide binds. These experiments were hampered by the difficulties in obtaining complete cleavage of the carboxylase by either enzymatic or chemical methods. Nevertheless, we were able to obtain an amino-terminal sequence from a small cyanogen bromide fragment of γ-glutamyl carboxylase cross-linked to pro-FIX19. The γ-glutamyl carboxylase was chemically cross-linked to 125I-labeled pro-FIX19 by disuccinimidyl suberate. The cross-linked γ-glutamyl carboxylase was then fractionated by reducing SDS-PAGE and transferred to a PVDF membrane. The location of the cross-linked carboxylase was determined by autoradiography, and it was excised from the membrane and cleaved in situ with cyanogen bromide. The resulting cyanogen bromide fragments were again fractionated by reducing SDS-PAGE and transferred to a PVDF membrane for sequence analysis. The primary sequence obtained from the amino terminus of this radioactive cyanogen bromide fragment was KDHADMLKQYATC, which corresponds to residues 438–450 of the γ-glutamyl carboxylase and is shown boxed in Fig. 3. In this case, cyanogen bromide cleavage occurred at a tryptophan rather than the expected methionine residue. Although not common, other examples of cleavage at tryptophan residues by cyanogen bromide have been reported (19). Secondary and tertiary sequences were also pres-

**Fig. 6.** Chemical cross-linking of the propeptide to the native and limited trypsinized γ-glutamyl carboxylase. The cross-linking conditions are indicated above the reducing SDS-PAGE silver-stained gel (upper) and its autoradiogram (lower). pro-FIX19 is the propeptide sequence of human factor IX, and FIXQ/S is the propeptide plus the Gla domain sequences of human factor IX. Thick and thin arrows on left, FIXQ/S and pro-FIX19 cross-linked fragment, respectively.

shows that cross-linking is observed when either pro-FIX19 or FIXQ/S is used for cross-linking and that cold pro-FIX19 effectively competes with either radioactive peptide. Fig. 6 also clearly demonstrates that the propeptide of factor IX binds to the carboxyl-terminal 60-kDa fragments as well as the 50-kDa fragment of the γ-glutamyl carboxylase molecule. This observation has been repeated many times with different preparations of γ-glutamyl carboxylase and with several different propeptides, including pro-FIX19, FIXQ/S, and biotin-pro-FIX19-biotin. Identical results were obtained whether the cross-linking was done before or after the limited trypsin digestion. The only requirement appears to be that the γ-glutamyl carboxylase is enzymatically active. The fact that only active carboxylase will cross-link to the propeptide is further evidence for the specificity of the cross-linking reaction.

During the course of this work Yamada et al. (16) published that the propeptide binding site was found within the aminoterminal 259 amino acids of the recombinant γ-glutamyl carboxylase. Because our results were different, we attempted to resolve the cause of the difference. A photochemical peptide, pro-FIX19-16BPA, with sequence identical to that used by Yamada et al. (16) was obtained and used for photochemical cross-linking. Fig. 7 demonstrates that pro-FIX19-16BPA is predominantly cross-linked to the 60-kDa fragment no matter whether the cross-linking was accomplished photochemically or was DSS-mediated. Fig. 7A depicts a silver-stained gel of the autoradiogram shown in Fig. 7B and demonstrates that both the 60- and 30-kDa fragments are present in our preparation. To confirm further that binding was predominately in the 60-kDa fragment, we trypsin-cleaved another carboxylase sam-

**Fig. 7.** SDS-PAGE gels of pro-FIX19-16BPA cross-linked to the carboxylase. Lane 1, molecular weight standards; lanes 2 and 3 are photochemically cross-linked; lanes 4 and 5 are cross-linked with DSS. Lanes 3 and 5, same as lanes 2 and 4 except for the presence of an excess of unlabeled pro-FIX19-16BPA. A, silver-stained gel; B, autoradiograph of the same gel.
ent in the sequence analysis. The secondary sequence resulted from cleavage after the methionine at 443, whereas the tertiary sequence was apparently the result of unexplained cleavage following histidine 440.

The radioactive band migrated as 16 kDa (Fig. 9); however, because it is cross-linked to pro-FIX19, which is 2.3 kDa, and there is a potential N-glycosylation site, we estimate that the size is considerably less than 16 kDa. The most reasonable assumption is that it terminates at residue 507, the next methionine in the carboxylase. Cleavage at the next methionine beyond methionine 507 (methionine 579) would be expected to give a 17.7-kDa fragment, which, if cross-linked, would certainly migrate as a larger fragment. This size would probably be even larger, because four of the nine potential N-glycosylation sites of the γ-glutamyl carboxylase are located between residues 438 and 579. The cyanogen bromide fragment that we isolated and sequenced appears to be the major species of labeled fragment, because there are no other radioactive bands visible on the gel. The 125I-proFIX19 used for cross-linking was also used for a size marker, making it very unlikely that any cross-linked peptides could have run off the end of the gel during PAGE analysis. Furthermore, three overlapping sequences obtained from the radioactive cyanogen bromide band are all found within the larger tryptic fragment that we identified by immunoblotting and amino acid sequencing.

Sugiura et al. (17) recently published a mutagenesis study on bovine carboxylase expressed in Chinese hamster ovary cells (17). They concluded from their studies that “lysin 217 or lysine 218 may be key for substrate and/or propeptide recognition or for catalysis.” Chinese hamster ovary cells contain endogenous carboxylase activity, and it is therefore difficult to interpret their kinetic data. Interpretation is especially difficult in the relevant mutants in which low activity levels of bovine carboxylase were encountered. It is of note, however, that they observed that higher propeptide concentrations were required to stimulate the incorporation of 14CO2 into FLEEL in the γ-glutamyl carboxylase mutated at positions 513 and 515, which would support the data presented in this article.

The differences in the results reported here and those reported by Yamada et al. (16) and confirmed by Sugiura et al. (17) are probably due to their implicit assumption that the migration of proteins on gels is directly proportional to their molecular mass. However, it is true that some regions of the two fragments are in very close proximity, as shown by the fact that the 60- and 30-kDa fragments are joined by a disulfide bond(s). Therefore, it is possible that lysines 217 and 218 are in close proximity to residues in the carboxyl half of the carboxylase molecule and that their conditions were sufficiently different from ours that, in their hands, the propeptide reacted with amino-terminal residues rather than the carboxyl-terminal residues that we saw. Although there is serious difficulty in interpreting some of the data of Sugiura et al. (17) because of the endogenous carboxylase activity, their data suggest that mutations at residues 234–235, 359–361, 406–408, and 513–515 all affect propeptide binding. It is difficult to draw firm conclusions until there is a more extensive characterization of their mutated enzymes in a system in which there is no endogenous γ-glutamyl carboxylase. But it appears that the data of Sugiura et al. (17) agree as well with our data as they do with the data of Yamada et al. (16).

Our data, derived from both cyanogen bromide and tryptic cleavage, demonstrate clearly that the propeptide is cross-linked to the carboxyl, luminal portion of the γ-glutamyl carboxylase. We conclude that the propeptide is cross-linked to residues between lysine 438 and methionine 507. Thus, this area is a prime candidate for further mutational analysis.

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