# The active site region of the vitamin K-dependent carboxylase includes both the amino-terminal hydrophobic and carboxy-terminal hydrophilic domains of the protein

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**Abstract In order to localize the active site of the vitamin K-dependent carboxylase, we developed an affinity probe containing the propeptide and the first two carboxylatable glutamate residues conserved in many native substrates. This probe crosslinked to both the hydrophobic amino-terminal and hydrophilic carboxy-terminal domains of the carboxylase, in contrast with previous work which localized both the catalytic and the propeptide binding site within the amino-terminal hydrophobic domain. Amino acid analysis revealed that the mass of an amino-terminal fragment is seriously underestimated by SDS-PAGE. Reanalysis of the published data in light of this information suggests that a portion of the propeptide binding site resides within the carboxy-terminal hydrophilic domain.**



*Key words:* Vitamin K-dependent carboxylase; Photolabeling; *p*-Benzoylphenylalanine; Enzyme inactivation; Catalytic site; Propeptide binding site

## 1. Introduction

The γ-glutamyl carboxylase is involved in the post-translational maturation process of vitamin K-dependent proteins such as blood clotting factors (Factors VII, FIX, FX and prothrombin), anti-clotting factors (proteins C and S), bone gla protein, bone matrix protein, protein Z and others [1–5]. During this maturation process, the γ-glutamyl carboxylase adds a second carboxyl group to selected glutamate residues localized near the amino terminus of those proteins. In the case of clotting factors, a propeptide region (−18 to −1) is a crucial element in substrate recognition by the γ-glutamyl carboxylase [6–8].

The γ-glutamyl carboxylase is a glycosylated membrane protein that has been purified to near homogeneity from bovine liver endoplasmic reticulum [9]. The first complete γ-glutamyl carboxylase sequence (758 amino acids) was deduced from the human cDNA [10] and the protein has been expressed with activity in several cell types [10–12]. The enzyme probably has 3 or 5 transmembrane spans in the hydrophobic amino-terminal part of the protein.

In order to determine the contributions of the hydrophobic and hydrophilic domains to the active site and substrate binding site, synthetic peptide probes designed to mimic the natural substrates have been crosslinked to the carboxylase. Using the affinity peptide-substrate inactivator, [$^{125}$I]tyrosyl-*N*-(bromoacetyl)-FLEELY, Kuliopulos et al. labeled a site in the amino-terminal part of the carboxylase [17] suggesting that the catalytic active site could reside in the amino-terminal hydrophobic domain of the enzyme. In order to identify the propeptide binding site Yamada et al. used [$^{125}$I]tyrosyl-proFIX19 analogs containing the photoactivatable residue, *p*-benzoylphenylalanine (Bpa) and labeled sites that were also localized in the amino-terminal hydrophobic part of the carboxylase [18]. This later result was confusing since bifunctional crosslinking reagents had crosslinked a propeptide probe to the carboxy-terminal hydrophilic domain [19,20].

Having contributed to the understanding of the catalytic mechanism [13–16], we are now turning to study the protein. In the present study we used a photactivatable affinity probe containing the factor IX propeptide sequence (−18 to −1) and a portion of prothrombin (+1 to +10) [21] to label the carboxylase (CP1, Table 1). The photoactivatable group, Bpa [22], was placed at position +4 close to the first carboxylatable glutamate residue (+6) in order to identify the catalytic active site of the carboxylase. An iodinated version of this probe, [$^{125}$I]CP2, labeled both an amino-terminal 30-kDa$_{app}$ trypsin fragment containing the hydrophobic domain and a carboxy-terminal 60-kDa$_{app}$ trypsin fragment containing the hydrophilic domain, suggesting that the active site region includes portions of both domains. Moreover, purification and amino acid analysis of the 60-kDa$_{app}$ fragment surprisingly indicated its mass was seriously underestimated by SDS-PAGE.

In previous studies [17,18] crosslinking sites were localized indirectly using fragment molecular mass estimation based on SDS-PAGE mobility. In light of this new information we reinterpreted the published data to obtain an internally consistent picture that suggests that a significant portion of the propeptide binding site is in the carboxy-terminal hydrophilic domain.

## 2. Materials and methods

### 2.1. Synthesis, purification and iodination of Bpa peptides

Solvents and reagents were used without further purification, except for triethylamine and trifluoroacetic acid which were distilled. The

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*Abbreviations:* Bpa, *p*-benzoylphenylalanine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; CP1 or 2, carboxylase probe 1 or 2 (see Table 1); kDa$_{app}$, apparent mass in kDa on SDS-PAGE; MOPS, 4-morpholinopropanesulfonic acid; NαBoc, αNH$_2$ protected by terbutoxycarbonyl group; PVDF, polyvinylidene fluorure; TCA, trichloroacetic acid


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NαBoc amino acids were purchased from Bachem, Neosystem, Novabiochem and Propeptide. The NαBoc Bpa was prepared[2] with an overall yield of 61%. Peptides (FLEEV, proFIX/PT28, CP1, CP2 and proFIX19) were prepared by the solid phase procedure with NαBoc amino acids using a ABI 431A synthesizer [25], with 0.1 mmol of PMA resin. The deprotected peptides were purified by RP-HPLC (purity superior to 95%). The molecular mass of the peptides was confirmed with mass spectrometry, by B. Renaud and J-C. Tabet (Université Pierre et Marie Curie). The $\lambda_{max}$ and ε ($M^{-1}$ $cm^{-1}$) were 264 nm and 11460 nm for CP1 and 264 nm and 16140 nm for [$^{127}$I]CP2.

The tyrosyl residue of CP2 (1.41 μmol) was iodinated with Na$^{127}$I (800 μl, 2.65 nM in 0.1 N NaOH) in a sodium acetate buffer (3 ml, 0.5 N, pH = 6) by addition of a chloramin T solution (235 μl, 9 mM) [26]. The reaction was stopped after 5 min by addition of a sodium thiosulfate solution (500 μl, 1.48 mg/ml). The 100% monoiodinated peptide was purified by RP-HPLC and the molecular mass confirmed with mass spectrometry (MALDI-TOF) by B. Renaud (Université Pierre et Marie Curie). The $^{125}$I iodination of CP2 was performed using iodo-beads according to manufacture instructions (Biorad). The specific activity of [$^{125}$I]CP2 was 184 Ci/mole.

*2.2. Carboxylase purification, assay and analysis*

Carboxylase was prepared from bovine liver using a proFIXQ/S affinity matrix as described [9,27]. The purified enzyme preparation used in this study contained between 4 and 30 nM carboxylase, 1.6 μM ProFIX19, 0.8% CHAPS, 0.1% PC, 25 mM MOPS (pH 7.4), 500 mM NaCl and 20% glycerol.

Incorporation of $^{14}CO_2$ into peptide substrates was measured in 125 μl reactions containing 56 μl of Buffer A (25 mM MOPS pH 7, 100 mM NaCl, 20% glycerol), peptide (20 μl), PC/CHAPS (4 μl, 5%/5%), ammonium sulfate (25 μl, 4 M), carboxylase (20 μl, 20 nM), Vitamin K hydroquinone (5 μl, 10.8 mM, 162 mM DTT) and Na$H^{14}CO_3$ (5 μl, 5 μCi). The reaction was started by the addition of Na$H^{14}CO_3$ at 20°C and stopped after 30 min by the addition of 50 μl of 30% TCA. Aliquots of 100 μl were then placed into a desiccator containing $P_2O_5$ and KOH overnight. Samples were taken up into 300 μl of water and 1 ml of Aquasol scintillation fluid and counted on a LKB 1214 Rack-beta liquid scintillation counter.

The carboxylase and the carboxylase fragments were analyzed on mini gels [28] which included a 4% stacking gel and a resolving gel with the acrylamide concentration indicated in the text. Following electrophoresis, gels were phosphoimaged immediately without fixing or drying.

*2.3. Photolabeling of γ-glutamyl carboxylase with Bpa peptides*

Prior to photoinactivation of the carboxylase with the Bpa peptides, the same volumes of Buffer A, carboxylase, PC/CHAPS and ammonium sulfate as used in the carboxylase assay were combined with Bpa peptides (5 μl, 150 μM) (total volume 100 μl) and preincubated for 30 min at 20°C. Samples in Eppendorf tubes were then placed in an ice bath and irradiated at 6 cm from a Phillips HPR 125 UV lamp for various times. Following irradiation each 100 μl reaction point received FLEEV (15 μl, 30 mM), Vitamin K hydroquinone (5 μl, 10.8 mM, 162 mM DTT) and Na$H^{14}CO_3$ (5 μl, 5 μCi). The carboxylation reaction was otherwise performed according to the standard assay.

For [$^{125}$I]CP2 labeling of purified carboxylase, the carboxylase was combined with 0.02 vol of 10% PC and 0.1 vol of [$^{125}$I]CP2 (6.5 μM except otherwise indicated). Samples were incubated for 30 min at 20°C prior to photoirradiation for 15 min in an ice bath as described above. Following irradiation, samples to be subjected to SDS-PAGE were combined with 4×SDS-PAGE sample buffer containing 40 mM DTT and run on 10% gels. Relative radioactivity incorporated into the carboxylase under various conditions was quantitated from the phosphoimage on a Molecular Dynamics Phosphoimager (Sunnyvale, CA). In some cases the incorporated radioactivity was determined directly by gamma counting. In competition experiments with proFIX/PT28, 0.2 vol of this peptide at various concentrations was added just prior to 0.1 vol of [$^{125}$I]CP2 (55 nM).

*2.4. Proteolytic analysis of [$^{125}$I]CP2-labeled carboxylase*

Labeled carboxylase was incubated with 0.03 vol of Trypsin (1.8 ng/μl) at 0°C for 1 h. For samples subjected to SDS-PAGE the reaction was terminated by addition of 2× sample buffer with 20 mM DTT. Large scale trypsin cleavage of [$^{125}$I]CP2-labeled carboxylase (60 ml, 30 nM) was performed in the same way except the trypsin reaction was terminated by the addition of 0.02 vol of 500 μg/μl Leupeptin. Aliquots of these digestions were frozen in liquid nitrogen and stored at −70°C.

To purify the 30 and 60-kDa$_{app}$ trypsin fragments, portions (10 ml) of labeled trypsinized carboxylase (~300 pmol) were thawed, combined with 1 ml of 10% SDS and loaded onto a 140 ml Sephacryl 300 column equilibrated with column buffer (20 mM MOPS, pH 7 and 0.1% SDS). The column was eluted with column buffer at room temperature. Fractions (2 ml) containing the labeled carboxylase (20 ml) were combined and concentrated using two Centriplus 3 concentrators (Amicon) at room temperature by overnight centrifugation at 3000×*g*. SDS-PAGE sample buffer which did not contain DTT was added to the concentrated carboxylase (100 to 500 μl) and this material was run on 10% gels. The labeled carboxylase was located on the gel and then cut out using both a phosphoimage of the unfixed gel and prestained markers. Gel slices containing the carboxylase were combined with an equal volume of 2×SDS-PAGE sample buffer containing 200 mM DTT and incubated for 2 h at room temperature. These slices and the equilibrated buffer were then placed onto a second gel which included a stacking gel twice the depth of the sample wells. Following electrophoresis gel slices containing separately the reduced 30 and 60-kDa$_{app}$ trypsin fragments were cut out as described previously. The fragments were sometimes combined with elution buffer (20 mM MOPS, pH 7.4, 0.1% SDS) and eluted into dialysis tubing (12 kDa cutoff) in elution buffer with 10 V/cm electric field for 1.5 h. Alternatively the fragments were digested 'in gel' as described below. During all these purification steps the recovery was estimated by gamma counting of the incorporated $^{125}$I.

For 'in gel' digestions, gel slices containing, respectively, the 30 and 60-kDa$_{app}$ fragments were frozen in liquid nitrogen and stored at −70°C. Immediately before use these slices were thawed, combined with 2×SDS-PAGE sample buffer containing 20 mM DTT and incubated for 60 min at room temperature. The slices were then placed at the bottom of stacking gel wells which were half as deep as the stacking gel. Protease was added to the equilibrating sample buffer and this was layered over the gel slice. The gel was run until the stacking front was 66% through the stacking gel at which time the power was shut off for 30 min After this digest incubation the samples were run through a 15% gel.

To obtain sequence information on the purified 60-kDa$_{app}$ fragment, the purified fragment was run on a 10% SDS acrylamide gel and transferred for 90 min at 70 V to PVDF Immobilon (Millipore) as recommended by Millipore. Sequence was determined with an Applied Biosystems 475A Protein Sequencer by Dr. Russel Henry at the Protein Chemistry Laboratory at UNC Chapel Hill/NIEHS.

## 3. Results

*3.1. CP1 as a substrate*

A hybrid peptide containing the propeptide sequence of Factor IX and the first 10 amino acids of prothrombin (proFIX/PT28), was chosen as the basis of our affinity probe because it is a carboxylase substrate with a low $K_m$ [21]. We incorporated the photoprobe, Bpa, at position +4 near the

Table 1
Peptides sequence

| Peptide | Sequence | | | |
|---|---|---|---|---|
| | −18 | −16 | +1 +4 | +10 |
| proFIX 19 | TVFLDHENANKILNRPKRY | | | |
| proFIX/PT28 | TVFLDHENANKILNRPKRANTFLEEVRK | | | |
| CP1 (carboxylase probe 1) | TVFLDHENANKILNRPKRANT**B**LEEVRK | | | |
| CP2 (carboxylase probe 2) | TVFLDHENANKILNRPKR**Y**NT**B**LEEVRK | | | |

**B**, Bpa.

[2] Using *p*-bromomethyl benzophenone according to [23,24].

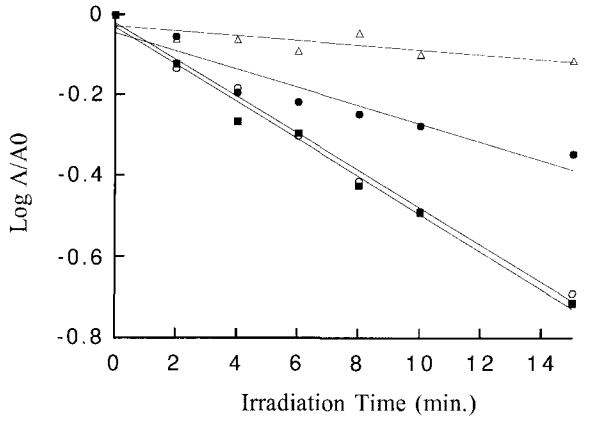


Fig. 1. Semi-logarithmic representation of the residual carboxylase activity versus UV irradiation time in the presence of the Bpa peptides. Carboxylase (0.4 pmol) was irradiated on ice without peptide inactivator (△) or with 7.5 μM CP1 (●), CP2 (○), or [$^{127}$I]CP2 (■). Residual carboxylase activity was assayed by measuring $^{14}CO_2$ incorporation into FLEEV as described in the Methods (see Section 2).

carboxylatable glutamates (+6 and +7) in order to identify residues near the catalytic active site of the carboxylase (Table 1). Under the assay conditions used, we found for this modified peptide, CP1 (carboxylase probe 1), had $K_{m,app}$ and $V_{m,app}$ values of 6.8 μM and $9.25\times10^6$ cpm $h^{-1}$ $mg^{-1}$, respectively. These values were similar to those of proFIX/PT28 (1.2 μM; $22.07\times10^6$ cpm $h^{-1}$ $mg^{-1}$). Thus the presence of the Bpa photoprobe at position +4 does not prevent association of CP1 with either the propeptide or glutamate binding sites in the carboxylase.

### 3.2. *Pseudo-first order inactivation of the γ-glutamyl carboxylase by CP1, CP2 and [$^{127}$I]CP2*

In order to follow the crosslinked photoaffinity ligand during proteolytic digestion of the carboxylase, an iodination site was introduced into CP1 by changing the alanine at position +1 to tyrosine to obtain carboxylase probe 2 (CP2). To demonstrate inactivation of the carboxylase by the photoaffinity ligands, the enzyme was photoirradiated on ice at 365 nm with CP1, CP2 and mono [$^{127}$I]CP2 for various times. The remaining carboxylase activity after photoirradiation was determined by measuring the incorporation of $^{14}CO_2$ into the pentapeptide FLEEV (3 mM) [29]. A 15 min irradiation in the absence of peptide resulted in a 10% inactivation of the carboxylase, whereas in the presence of proFIX/PT28 (Bpa +4) and CP2 or its iodinated derivative 56% and 80% inactivation were, respectively, observed (Fig. 1). For all three peptides, the semi-logarithmic representation of the residual activity versus UV irradiation time was linear, demonstrating a pseudo-first order inactivation of the γ-glutamyl carboxylase by an irreversible process.

### 3.3. *[$^{125}$I]CP2 labeling of the γ-glutamyl carboxylase*

Irradiation of the γ-glutamyl carboxylase in the presence of [$^{125}$I]CP2 resulted in incorporation of $^{125}$I into a band corresponding to the purified carboxylase on SDS polyacrylamide gel (data not shown). Photolabeling at several probe concentrations (Fig. 2) showed saturation behaviour with near maximal labeling at a concentration of 1 μM for a 15 min irradiation. In agreement with the nearly complete inactivation of the carboxylase under these conditions, quantitation of the incorporated label indicated a labeling efficiency around 90%.

In order to demonstrate that [$^{125}$I]CP2 was specifically associating with the active site region of the γ-glutamyl carboxylase, photocrosslinking was carried out in presence of increasing concentration of proFIX/PT28. As shown in Fig. 3, proFIX/PT28 at concentrations above 10 μM prevented most [$^{125}$I]CP2 crosslinking.

### 3.4. *Proteolytic digestion of the [$^{125}$I]CP2-labeled carboxylase*

Limited tryptic digestion of the carboxylase yielded two disulfide-linked fragments with molecular masses of 30 and 60 $kDa_{app}$, corresponding, respectively, to the amino and carboxy-terminal part of the γ-glutamyl carboxylase (see below). Limited tryptic digestion of [$^{125}$I]CP2-labeled carboxylase revealed radioactive labeling of both the 30 and 60-$kDa_{app}$ bands (Fig. 4). About 60% of the total labeling were found in the amino-terminal 30-$kDa_{app}$ fragment, while the remain-

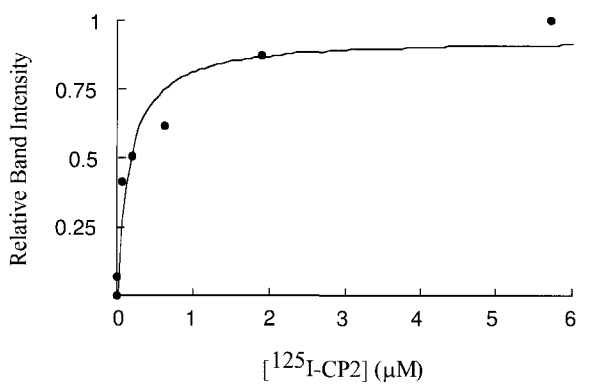


Fig. 2. Carboxylase labeling as a function of [$^{125}$I]CP2 concentration. Carboxylase was irradiated for 15 min on ice with various concentration of [$^{125}$I]CP2 (2.6, 7.8, 70, 211, 634, 1900 and 5700 nM) as described in the Methods (see Section 2). Following photoirradiation, aliquots (40 μl containing 0.11 pmol of carboxylase) were run on a 10% gel and imaged with a phosphoimager. Quantitation of the relative [$^{125}$I]CP2 incorporation by phosphoimage analysis shows the saturable nature of this labeling.

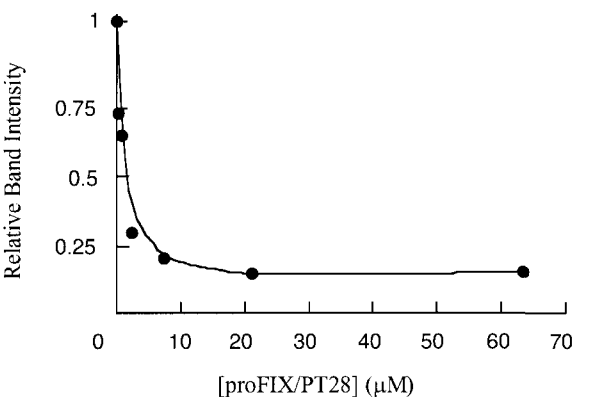


Fig. 3. ProFIX/PT28 blocks incorporation of [$^{125}$I]CP2 into the carboxylase. Carboxylase was irradiated for 15 min on ice with [$^{125}$I]CP2 (55 nM) and various concentrations of proFIX/PT28 (0.26, 0.78, 2.35, 7.5, 21.16 and 63.5 μM) as described in the Methods (see Section 2). Samples (46 μl containing 0.090 pmol of carboxylase) were run on a 10% gel. Relative incorporation of [$^{125}$I]CP2 into the carboxylase at the various proFIX/PT28 concentrations was quantitated from the phosphoimage of the gel.

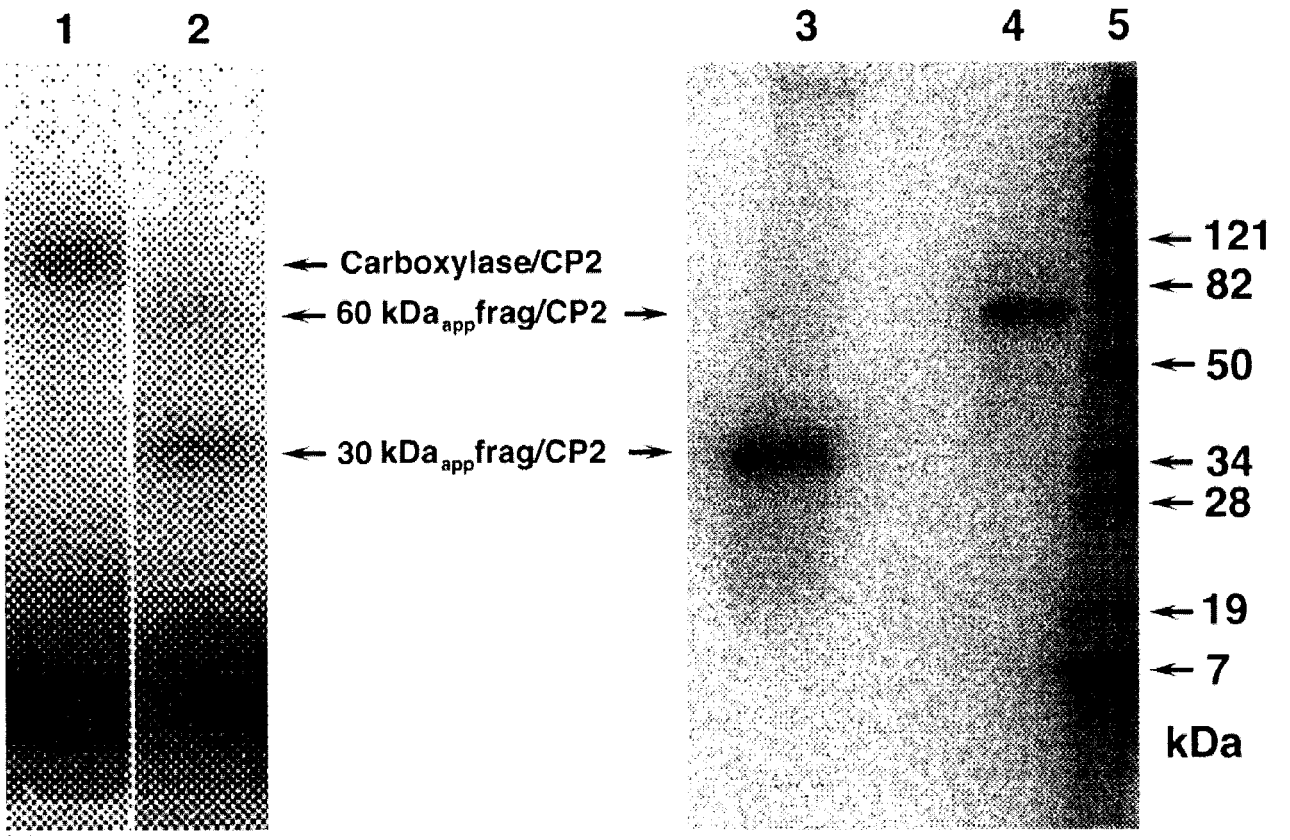


Fig. 4. [$^{125}$I]CP2 crosslinks to both the 30 and 60-kDa$_{app}$ trypsin fragments. Carboxylase (18 nM) crosslinked to [$^{125}$I]CP2 was subjected to limited trypsin (2 ng/μl) digestion for 1 h at 0°C. Following addition 2× sample buffer with 20 mM DTT, samples (50 μl) of crosslinked (lane 1) and crosslinked trypsinized (lane 2) carboxylase were run on 10% gel and phosphoimaged. Purified labeled 30 and 60-kDa$_{app}$ trypsin fragments are shown in lanes 3 and 4, respectively. Fragments crosslinked to [$^{125}$I]CP2 ran 5 kDa higher than uncrosslinked fragments.

ing 40% were found in the carboxy-terminal 60-kDa$_{app}$ fragment. Cleavage of carboxylase that had been labeled in the presence of increasing proFIX/PT28 showed that protection from [$^{125}$I]CP2 labeling was the same for the 30 and 60-kDa$_{app}$ fragments (data not shown).

As a first step toward identifying the [$^{125}$I]CP2 crosslinking sites, we developed a preparative technique for purifying the labeled 30 and 60-kDa$_{app}$ fragments. Following photolabeling and trypsinization, phospholipid and other contaminants were separated from the carboxylase by S-300 size exclusion chromatography in the presence of SDS. After concentration the SDS solubilized carboxylase was initially run on a non-reducing preparative SDS gel. Gel slices containing the trypsin cleaved but disulfide-linked 30 and 60-kDa$_{app}$ fragments were cut out, equilibrated with DTT containing sample buffer and run under reducing conditions on a second preparative gel. The now separated 30 and 60-kDa$_{app}$ fragments were cut out of the gel and subjected to proteolysis either 'in gel' or after elution into dialysis tubing.

Both the 30 and 60-kDa$_{app}$ fragments were used in attempts to obtain smaller fragments of the carboxylase that still retained the [$^{125}$I]CP2 peptide label. Unfortunately, various problems prevented more localized identification of the crosslinking sites. The 30-kDa$_{app}$ fragment, which contains the major crosslinking site(s), was found to be resistant to further proteolytic cleavage. This domain was resistant to cleavage even by the non-specific protease, proteinase K, however at concentrations 10-fold higher than required to digest other proteins, proteinase K did generate an incompletely digested smear of fragments unsuitable for sequencing (data not shown).

The 60-kDa$_{app}$ fragment which contains the minor crosslinking site was effectively cleaved by endo Glu-C into a family of discrete fragments (Fig. 5) including relatively stable 10 and 34-kDa$_{app}$ fragments. Unfortunately, we have been unable to identify the fragment sequence at the crosslinking site despite repeated attempts. The difficulty was mainly due to the small quantity of protein available.

In the process of localizing the crosslinking sites, the purified 60-kDa$_{app}$ fragment was sequenced following transfer to an Immobilon PVDF membrane. This analysis showed that the amino terminus of the carboxy-terminal 60-kDa$_{app}$ fragment is either R350 or R352 of the 758 amino acid γ-glutamyl carboxylase. Thus the amino-terminal 30-kDa$_{app}$ fragment has an actual protein molecular mass of 39 kDa and the carboxy-terminal 60-kDa$_{app}$ fragment has a protein molecular mass of 48 kDa.

## 4. Discussion

We designed the probe, [$^{125}$I]CP2, to label the catalytic site of the γ-glutamyl carboxylase by placing the photoprobe, Bpa, at position +4 near the carboxylatable glutamate residues (positions +6 and +7). Placement of the photoprobe in this position did not significantly decrease carboxylation compared to proFIX/PT28, indicating that the carboxylatable glu-

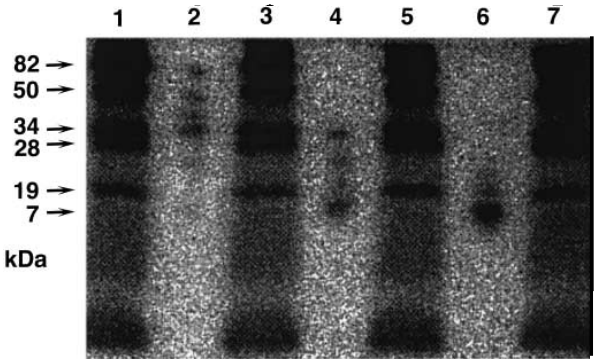


Fig. 5. Endo Glu-C digestion of the [$^{125}$I]CP2 crosslinked 60-kDa$_{app}$ trypsin fragment. The 60-kDa$_{app}$ trypsin fragment (~1000 cpm) was digested 'in gel' with 2.5 ng (lane 2), 50 ng (lane 4) and 1000 ng (lane 6) of endo Glu-C. Lanes 1, 3, 5 and 7 contain iodinated molecular weight markers of the indicated mass.

tamate residues were still associating with the catalytic site. Irreversible UV crosslinking of [$^{125}$I]CP2 to the carboxylase at different probe concentrations, was saturable, which is consistent with the peptide association at the carboxylase propeptide binding site. In further support of specific active site crosslinking, photolabelling by our affinity ligand approached 90% after a 15 min irradition, resulting in the incorporation of about 1 [$^{125}$I]CP2 per carboxylase. In total, these observations indicate that the carboxylatable glutamate residues could associate with the catalytic active site and bring the photoprobe into close proximity to the active site. Interestingly, [$^{125}$I]CP2 labeled both an amino-terminal 30-kDa$_{app}$ and a carboxy-terminal 60-kDa$_{app}$ trypsin fragment. Both of these associations were very likely specific, since the competitor proFIX/PT28 (10 μM) blocked labeling of each fragment with equal efficiency.

In order to interpret this observation, we localized the trypsin cleavage site by sequencing the 60-kDa$_{app}$ fragment. Unexpectedly, the amino terminus of this fragment was found to be at either R350 or R352 indicating that the carboxy-terminal 60-kDa$_{app}$ fragment had an actual protein molecular mass of 48 kDa. This cleavage location also shows that the amino-terminal 30-kDa$_{app}$ fragment has an actual protein molecular mass of 39 kDa. The 30-kDa$_{app}$ fragment includes the amino-terminal hydrophobic domain and 60-kDa$_{app}$ fragment contains most of the carboxy-terminal hydrophilic domain (Fig. 6). The fact that [$^{125}$I]CP2 labeled both fragments suggests that the active site region includes portions of both domains.

The anomalous migration of the 30-kDa$_{app}$ fragment may explain the discrepancy between the observations of Wu et al. [19] and Yamada et al. [18] who tentatively localized the propeptide binding site, respectively, in the carboxy-terminal hydrophilic and amino-terminal hydrophobic domains of the carboxylase. In their effort to localize this propeptide recognition site Yamada et al. used [$^{125}$I]tyrosyl-proFIX19 analogs containing the photoprobe Bpa [18]. The binding sites for two of these probes were localized to amino-terminal 30 and 33-kDa$_{app}$ endo Glu-C fragments. Based on mass estimation from SDS-PAGE these cleavage sites were suggested to be near residue 225 (Fig. 6). However, these amino-terminal 30 and 33-kDa$_{app}$ endo Glu-C fragments probably display the highly anomalous SDS-PAGE migration observed for the amino-terminal 30-kDa$_{app}$ trypsin fragment (actual mass 39 kDa). Thus, it is likely that these endo Glu-C fragments are cleaved near the trypsin site (R350/352) in the carboxy terminal hydrophilic domain. This is made more likely by the fact that there are no cleavage sites between amino acids 249 and 313 at the end of the hydrophobic region. Because the [$^{125}$I]tyrosyl-proFIX19 crosslinking sites on the endo Glu-C fragments were localized very near the cleavage sites [18], these crosslinking sites are probably in the carboxy-terminal hydrophilic domain.

The original localization of the propeptide binding site within the amino terminal 225 amino acids of the carboxylase also did not agree well with the mutagenesis studies [18,12]. Of the three mutated patches that resulted in reduced carboxylase activity apparently because of poor propeptide binding, two were at residues 406/408 and 513/515 [12].

For reasons that are unclear, we could not precisely localize the [$^{125}$I]CP2 crosslinking site in the carboxy-terminal hydrophilic domain. However, endo Glu-C cleavage of the 60-kDa$_{app}$ trypsin fragment releases a relatively stable labeled 34-kDa$_{app}$ fragment. The stability of this fragment suggests the [$^{125}$I]CP2 crosslinking site is between residues 350 and 518 (Fig. 6), a span which includes only 4 cleavage sites, rather than between residues 518 and 758, a span which includes 28 cleavage sites. Although [$^{125}$I]CP2 was designed to label the catalytic active site, it is possible that crosslinking in the carboxy-terminal hydrophilic domain represents crosslinking at the propeptide binding site given the proximity of the photoprobe (Bpa, position +4) to the propeptide (−18 to −1). In total, the available evidence [18–20] suggests that a significant portion of the propeptide recognition site resides within the carboxy-terminal hydrophilic domain.

The fact that the Bpa residue in [$^{125}$I]CP2 is near the first diglutamate pair and that most of the labeling was associated with the hydrophobic amino terminal trypsin fragment is consistent with a catalytic active site that may be mostly located within this domain. This would agree with the assigned location for the [$^{125}$I]tyrosyl-*N*-(bromoacetyl)-FLEELY binding

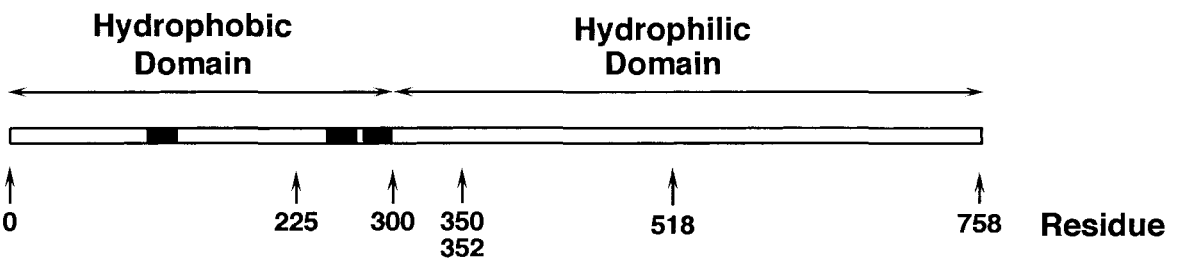


Fig. 6. Schematic representation of the vitamin K-dependent carboxylase sequence. The three putative transmembrane domains originally suggested [10] are indicated by the filled areas. The trypsin cleavage site (R350/R352) and likely [$^{125}$I]CP2 crosslinking location (between residues R350/R352 and E518) are indicated.

site [17]. The presence of a portion of the catalytic site within the hydrophobic domain is also suggested by mutagenesis experiments which showed that neutralization of the charged patch at residues 217/218 resulted in a mutant with no activity [12]. These results suggest that at least a portion of the catalytic active site resides in the hydrophobic domain.

In light of the anomalous migration of the amino terminal hydrophobic domain, the available evidence suggests that a significant portion of the propeptide recognition site resides in the hydrophilic domain probably in residues near the centre of the carboxylase. The fact that [$^{125}$I]CP2, designed to crosslink near the catalytic active site was associated with both the amino and carboxy-terminal trypsin fragments suggests the active site region includes elements of both the hydrophobic and hydrophilic domains.

## References

[1] Wright, D.J., Morris, D.P. and Stafford, D.W. (1995) in: Molecular Basis of Thrombosis and Hemostasis (High, K.A., and Roberts, H.R., Eds.), pp. 309–329, Marcel Dekker, Inc., New York.
[2] Suttie, J.W. (1993) FASEB J. 7, 445–452.
[3] Suttie, J.M. (1988) Biofactors 1, 55–60.
[4] Price, P.A. (1988) Annu. Rev. Nutr. 8, 565.
[5] Suttie, J.W. (1985) Annu. Rev. Biochem. 54, 459–477.
[6] Pan, L.C. and Price, P.A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 6109–6113.
[7] Jorgensen, M.J., Cantor, A.B., Furie, B.C., Brown, C.L., Shoemaker, C.B. and Furie, B. (1987) Cell 48, 185–191.
[8] Foster, D.C., Rudinski, M.S., Schach, B.G., Berkner, K.L., Kumar, A.A., Hagen, F.S., Sprechner, C.A., Insley, M.Y. and Davie, E.W. (1987) Biochemistry 26, 7003–7011.
[9] Wu, S.-M., Morris, D.P. and Stafford, D.W. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 2236–2240.
[10] Wu, S.-M., Cheung, W.F., Frazier, D.F. and Stafford, D.W. (1991) Science 254, 1634–1636.
[11] Roth, D.A., Rehemtulla, A., Kaufman, R.J., Walsh, C.T., Furie, B. and Furie, B.C. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 8372–8376.
[12] Sugiura, I., Furie, B., Walsh, C.T. and Furie, B.C. (1996) J. Biol. Chem. 271, 17837–17844.
[13] Dubois, J., Gaudry, M., Bory, S., Azerad, R. and Marquet, A. (1983) J. Biol. Chem. 258, 7897.
[14] Decottignies-Le Marechal, P., Ducrocq, C., Marquet, A. and Azerad, R. (1984) J. Biol. Chem. 259, 15010.
[15] Vidal-Cros, A., Gaudry, M. and Marquet, A. (1990) Biochem. J. 260, 749.
[16] Dubois, J., Dugave, C., Bory, S., Gaudry, M. and Marquet, A. (1991) Biochemistry 30, 10506.
[17] Kuliopulos, A., Nelson, N.P., Yamada, M., Walsh, C.T., Furie, B., Furie, B.C. and Roth, D.A. (1994) J. Biol. Chem. 269, 21364–21370.
[18] Yamada, M., Kuliopulos, A., Nelson, N.P., Roth, D.A., Furie, B., Furie, B.C. and Walsh, C.T. (1995) Biochemistry 34, 481–489.
[19] Wu, S.-M., Morris, D.P. and Stafford, D.W. Enzymes: Mechanisms of Action III A1414, 904.
[20] Wright, D.J., Morris, D.P. and Stafford, D.W. (1995) in: Molecular Basis of Thrombosis and Hemostasis (High, K.A., and Roberts, H.R., Eds.), pp. 323–324, Marcel Dekker, Inc., New York.
[21] Hubbard, B., Jacobs, M., Ulrich, M., Walsh, C.T., Furie, B. and Furie, B.C. (1989) J. Biol. Chem. 264, 14145–14150.
[22] Kauer, J.C., Erickson-Viitnamin, S., Wolfe, H.R. and Degrado Jr., W.F. (1986) J. Biol. Chem. 261, 10695–10700.
[23] Josien, H., Martin, A. and Chassaing, G. (1991) Tetrahedron Lett. 32, 6547–6550.
[24] Josien, H. and Chassaing, G. (1992) Tetrahedron Asym. 3, 1351–1354.
[25] Merrifield, R.B. (1963) J. Am. Chem. Soc. 85, 2149–2154.
[26] Heald, S.L., Jeffs, P.W., Lavin, T.N., Nambi, P., Lefkowitz, R.J. and Caron, M.G. (1983) J. Med. Chem. 26, 832–838.
[27] Morris, D.P., Soute, B.A.M., Vermeer, C. and Stafford, D.W. (1993) J. Biol. Chem. 268, 8735–8742.
[28] Laemmli, U.K. (1970) Nature 227, 1940.
[29] Suttie, J.W., Hageman, J.M., Lehrman, S.R. and Rich, D.H. (1976) J. Biol. Chem. 251, 5827–5830.