Localization of the Factor IX Propeptide Binding Site on Recombinant Vitamin K Dependent Carboxylase Using Benzoylphenylalanine Photoaffinity Peptide Inactivators†

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ABSTRACT: The propeptide binding/activation site on the vitamin K dependent carboxylase has been localized to a region of carboxylase between residues Arg +50 and Glu +225 by photoinactivation studies using [^{125}I]tyrosyl-labeled benzoylphenylalanine (Bpa)-containing analogs of proFIX19, a peptide containing residues −18 to +1 of factor IX. Four proFIX19 analogs with Bpa substituents at −16, −13, −7, and −6 were synthesized. These peptides were specific photoinactivators of carboxylase and were used to label a His_6-carboxylase construct produced in baculovirus-infected insect cells. Fragments of the labeled carboxylase produced by V8 protease digestion were analyzed by peptide-specific antibodies and by autoradiography. The propeptide recognition site was localized to the N-terminal one-third of the 94 kDa carboxylase. This is consistent with previous studies using a carboxylase substrate affinity label, *N*-(bromoacetyl)-FLEELY [Kuliopulos, A., Nelson, N. P., Yamada, M., Walsh, C. T., Furie, B., Furie, B. C., & Roth, D. A. (1994) *J. Biol. Chem. 269*, 21364−21370], indicating that the propeptide binding site and the FLEEL binding site are both located within the N-terminal one-third of the vitamin K dependent carboxylase.

The vitamin K dependent γ-glutamyl carboxylase is a key enzyme in the secretory compartment of liver cells for posttranslational carboxylation of up to 12 glutamyl residues in the first 50 residues of proteins that function in hemostasis, such as factor IX, factor X, and prothrombin (Furie & Furie, 1988). The posttranslational modification of Glu residues to γ-carboxyglutamyl (Gla)[1] residues, generating high-affinity Ca^{2+} sites, is crucial for the function of these coagulation proteins and their conversion from zymogens to active proteases on membranes (Nelsestuen et al., 1974; Stenflo, 1974; Esmon et al., 1975).

The carboxylase is unique in its requirement for dihydrovitamin K_1 (KH_2) for CO_2 fixation and also for O_2 consumption in each catalytic event, as vitamin K epoxide and H_2O are produced (Suttie, 1985, 1993). Also remarkable has been the finding that a propeptide region, e.g., residues −18 to −1, which subsequently was removed proteolytically, is a crucial recognition determinant that instructs carboxylase to act on glutamyl residues immediately downstream of the propeptide region (Pan & Price, 1985; Jorgensen et al., 1987; Foster et al., 1987; Ulrich et al., 1988; Hubbard et al., 1989; Huber et al., 1990), hence the specificity for carboxylation, in liver cells, of only amino proximal glutamyl residues in mature factor VII, factor IX, factor X, prothrombin, protein C, and protein S.

After much travail due to its hydrophobic nature and microsomal membrane association, bovine carboxylase was purified sufficiently using a 59 amino acid propeptide fragment of factor IX as an affinity ligand to enable cloning and sequencing (Wu et al., 1991a,b; Kuliopulos et al., 1992a; Rehemtulla et al., 1993). The human cDNA has also been sequenced (Wu et al., 1991a). Expression of the 758 amino acid carboxylase in mammalian cells leads to increased enzyme activity (Wu et al., 1991a; Rehemtulla et al., 1993), while expression of the enzyme, including a His_6 fusion construct, in insect cells has been particularly useful given the absence of any endogeneous activity (Roth et al., 1993; Kuliopulos et al., 1994).

To date, little information has been available about the location of the carboxylase active site or the key propeptide recognition site. We have recently reported that *N*-(bromoacetyl)-FLEELY is both a carboxylation substrate and an irreversible inactivator with a partition ratio of 30 turnovers/inactivation event (Kuliopulos et al., 1992a). Further, studies with [^{125}I]tyrosyl-*N*-(bromoacetyl)-FLEELY and His_6-carboxylase produced a radiolabeled, inactive carboxylase

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[1] Abbreviations: carboxylase, vitamin K dependent γ-glutamyl carboxylase; Bpa, benzoylphenylalanine; Gla, γ-carboxyglutamate; KH_2, dihydrovitamin K_1; vitamin K epoxide, *trans*-phytyl-2,3-epoxyvitamin K; HPLC, reverse-phase high-performance liquid chromatography; FLEEL, Phe-Leu-Glu-Glu-Leu; proPT18, peptide comprising residues HVFLAPQQARSLLQRVRR of prothrombin; proFIX19, peptide comprising residues TVFLDHENANKILNRPKRY of factor IX; FIXQS, recombinant peptide comprising residues −18 to 41 of profactor IX with mutations T18A, R4Q, R1S, M19I; IDA, iminodiacetic acid; T7-Ab, N-Ab, and C-Ab, antibodies directed against His_6-carboxylase residues −23 to −13, 86−99, and 661−673, respectively; V8 protease, endoproteinase Glu-C (*S. aureus* V8 protease); γ-CRS, γ-carboxylation recognition site.

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which, on partial proteolysis and epitope-specific antibody analysis, led to labeling of the amino one-third of the 94 kDa carboxylase as a first indication of active site location (Kuliopulos et al., 1994).

As noted earlier, it is not actually the sequence around glutamyl residues that determines whether they will be carboxylated, but it is how far downstream they are from the amphipathic helical propeptide region (Sanford et al., 1991) that determines fitness as a substrate. For example, the 18-residue proPT18 (−18 to −1) binds with a K_D of ca. 3−4 μM (Ulrich et al., 1988), while the substrate FLEEL (+4 to +8) has a K_m in the 1−10 mM range (Suttie, 1985; Vermeer, 1990). To begin to map the high-affinity propeptide recognition domain, we have applied an aryl ketone photoactivation strategy using benzoylphenylalanine (Bpa) as the photoprobe (Dormán & Prestwich, 1994; Shoelson et al., 1993; Williams & Shoelson, 1993) by synthesizing four Bpa-containing proFIX19 (residues −18 to +1 of human factor IX) peptides and demonstrating that these propeptides still activate the carboxylation of FLEEL. The photoinactivation by [^{125}I]tyrosyl-Bpa−propeptides yielded samples of inactive, labeled carboxylase that were digested by V8 protease and analyzed by epitope-selective antibodies.

MATERIALS AND METHODS

Synthesis, Purification, and Characterization of Bpa Propeptides. Solvents, solid support resin, and reagents were purchased from Applied Biosystems. Fmoc-L-amino acid derivatives were purchased from Peninsula Laboratories, and Fmoc-Bpa was from Bachem Bioscience Inc. Syntheses of factor IX Bpa propeptides (proFIX19-16Bpa, proFIX19-13Bpa, proFIX19-7Bpa, and proFIX19-6Bpa) were kindly performed by Charles Dahl at Harvard Medical School by the solid-phase procedure with Fmoc-amino acids, using a Milligen/Biosearch 9600 Synthesizer (Merrifield, 1965). The crude deprotected peptides were purified by reverse-phase high-performance liquid chromatography (HPLC) using a 21.5 mm × 25 cm Bio-Rad Hi-Pore 318 C_{18} reverse-phase column and a Waters high-performance liquid chromatography system. A flow rate of 5 mL/min and a linear gradient (0.4%/min) of acetonitrile/0.1% trifluoroacetic acid (TFA) in distilled, deionized water/0.1% TFA were employed. Peptide peaks were detected by UV absorbance at 214 nm. Fractions containing the desired peptides were combined and lyophilized. Reinjection of the purified peptides shwoed them to be >95% homogeneous. The sequences of the Bpa peptides were confirmed by laser desorption mass spectrometry (Biemann, 1989). The FLEEL pentapeptide was from Sigma and used without further purification.

Determination of Kinetic Parameters for Bpa Propeptides. The initial velocity of carboxylation of the FLEEL peptide was measured in the reaction mixtures of 125 μL systems with 2.7 mg (25 μL) of partially purified ammonium sulfate-precipitated bovine carboxylase (Kuliopulos et al., 1992a), 0.88 mM dihydrovitamin K_1, 1.5 mM $NaH^{14}CO_3$, and 10 μM Bpa containing propeptides in 20 mM sodium phosphate/150 mM NaCl (PBS) buffer including 0.8 M ammonium sulfate. These experiments were carried out under previously reported conditions (Kuliopulos et al., 1992a). K_m and V_m values for FLEEL were determined by a Wilkinson (1961) hyperbolic weighted least-squares analysis. The K_i value of proFIX19-16Bpa was obtained by inhibition of carboxylation of proPT28 (HVFLAPQQARSLLQRVRRANTFLEEVRK) (Ulrich et al., 1988).

Photoaffinity Inactivation of Bovine Carboxylase with Bpa Propeptides. The wild-type bovine carboxylase used in inactivations was the ammonium sulfate-precipitated microsomal preparation as described earlier. Photoaffinity inactivation of carboxylase by Bpa peptides was performed on ice in 60 μL volumes containing 30 μL of ammonium sulfate-precipitated microsomes (AS carboxylase) and 40 μM Bpa propeptide in PBS. For control experiments, proPT18 or proFIX19 was used instead of Bpa peptide. The photoaffinity inactivation reactions were carried out in sealed 100 μL polystyrene dishes on ice, and the samples were irradiated for 15 min at a distance of 20 cm from the light source, using a focused HBO 100 W mercury short arc lamp with a filter in place to eliminate any light below 340 nm [method described by Y.-M. Li et al. (unpublished data)]. In order to assay for remaining carboxylase activity, 25 μL aliquots were removed and diluted into 100 μL of an assay solution which, when diluted with an additional 25 μL per assay reaction, contained 10 μM FIXQS, 0.88 mM KH_2, and 1.5 mM $NaH^{14}CO_3$ (10 μCi) in PBS. The individual assay reactions were incubated at 25 °C for 30 min and quenched under previously reported conditions (Kuliopulos et al., 1992a).

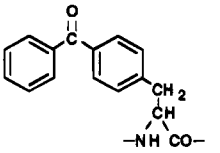
Tyrosine Iodination and Purification of Bpa Propeptides. The iodination of tyrosyl residues was performed by the chloramine-T method (Hunter & Greenwood, 1962) as modified by Kuliopulos et al. (1992a). The specific activity of proFIX19-16Bpa[^{125}I]Y was 2.1Ci/μmol (640 μCi, yield 64%). Iodinations of proFIX19-13Bpa, proFIX19-7Bpa, and proFIX19-6Bpa were carried out using the same method as for proFIX19-16Bpa, and yields were as follows: proFIX19-13Bpa[^{125}I]Y, 66%; proFIX19-7Bpa[^{125}I]Y, 53%; proFIX19-6Bpa[^{125}I]Y, 60%.

Production of His_6-Carboxylase. A His_6-tagged bovine liver carboxylase (His_6-carboxylase) was produced in insect cells using a baculovirus expression system (Roth et al., 1993), and microsomal preparations were carried out as described previously (Kuliopulos et al., 1994).

Photoaffinity Inactivation of His_6-Carboxylase with Bpa Propeptides and Purification of Bpa Propeptide-Inactivated Carboxylase. In a typical experiment, the dried proFIX19-16Bpa[^{125}I]Y peptide (9.5 pmol, 20 μCi) and 166 μL of microsomal His_6-carboxylase (specific activity of 4.9×10^6 dpm h^{-1} mg^{-1}; Roth et al., 1993; Kuliopulos et al., 1994) were incubated in PBS (150 mM NaCl/20 mM sodium phosphate, pH 7.4) containing 0.8 M ammonium sulfate for 10 min at room temperature in the dark. After the incubation, the mixture was irradiated for 15 min at 350 nm and 4 °C, as described earlier, and then quenched with 130 mg of urea.

The irradiated mixture was purified by Ni chelation chromatography as described previously, except that a 4 mL iminodiacetic acid (IDA)−Sepharose column was used (Kuliopulos et al., 1994). The yield of His_6-carboxylase−proFIX19-16BPa[^{125}I]Y was 0.62% based on starting [^{125}I]. The photo-inactivation reactions using proFIX19-13Bpa-[^{125}I]Y, proFIX19-7Bpa[^{125}I]Y, and proFIX19-6Bpa[^{125}I]Y were carried out as for proFIX19-16Bpa[^{125}I]Y. The yields were as follows: His_6-carboxylase−proFIX19-13Bpa[^{125}I]Y, 0.36%; His_6-carboxylase−proFIX19-7Bpa[^{125}I]Y, 0.44%; His_6-carboxylase−proFIX19-6Bpa[^{125}I]Y, 0.31%. The His_6-carboxylase−proFIX19-16BPA[^{125}I]Y eluted from the IDA−Sepharose column was purified further by preparative polyacrylamide gel electrophoresis (Kuliopulos et al., 1994).

	-18 propeptide -1+1
ProFIX19	TVFLDHENANKILNRPKRY
ProFIX19-16BPA	TV***B***LDHENANKILNRPKR<u>Y</u>
ProFIX19-13BPA	TVFLD***B***ENANKILNRPKR<u>Y</u>
ProFIX19-7BPA	TVFLDHENANK***B***LNRPKR<u>Y</u>
ProFIX19-6BPA	TVFLDHENANKI***B***NRPKR<u>Y</u>
ProPT18	HVFLAPQQARSLLQRVRR



B : Bpa (Benzoylphenylalanine)

<u>Y</u> : ^{125}I-Tyrosine

FIGURE 1: Sequences of carboxylase substrate propeptides encompassing the propeptide domains of profactor IX, Bpa-containing profactor IX, and prothrombin. *B* designates a Bpa residue, and <u>Y</u> designates a [^{125}I]tyrosine residue.

Proteolytic Digestion of Bpa Propeptide-Inactivated Carboxylase. Aliquots of pure His_6-carboxylase−proFIX19-16BPA[^{125}I]Y were digested with varying concentrations of endoproteinase Glu-C (*S. aureus* V8 protease) in 100 mM NH_4HCO_3 (pH 8.0) and 0.1% SDS for 1 h. The proteolyzed material was electrophoresed in precast 4−20% SDS−PAGE gels (Bio-Rad) and electroblotted onto PVDF membranes. Western blot analysis of the membranes was performed by using three regiospecific antibodies [a T7-tag monoclonal antibody against the 11 amino acid bacteriophage T7 gene 10 leader peptide, incorporated into His_6-carboxylase fusion protein residues−23 to −13 (T7-Ab); an N-terminal rabbit polyclonal antipeptide antibody directed against residues 86−99 (N-Ab); and a C-terminal rabbit polyclonal antipeptide antibody directed against residues 661−673 (C-Ab)], followed by enhanced chemiluminescence as described previously (Kuliopulos et al., 1994).

RESULTS

Rate Enhancement of Carboxylase with Bpa-Containing Factor IX Propeptides. Vitamin K carboxylase specifically interacts with the propeptide region of the precursor forms of vitamin K dependent proteins. These propeptides have high sequence homology, and NMR studies show that proPT18 potentially forms an amphipathic α-helix (Sanford et al., 1991). We made four novel Bpa-containing propeptide analogs for photoaffinity inactivation in order to identify the region of the carboxylase that binds propeptide. We selected proFIX19 as the model propeptide and used the tyrosine residue at +1 for radioiodination (Figure 1). Substitutions were performed at positions Phe −16, Pro −13, Ile −7, and Leu −6, replacing each with a Bpa residue (Figure 1) because the residues −18 to −15 and −10 or less (certainly −6) of the propeptide are particularly important for recognition of the carboxylase (γ-CRS; Pan & Price, 1985; Jorgensen et al., 1987; Ulrich et al., 1988; Cheung et al., 1990; Hubbard et al., 1989; Huber et al., 1990). These residues may form a hydrophobic face that is likely to be involved in recognition by carboxylase (Sanford et al., 1991).

These Bpa peptides were conveniently synthesized by a solid-phase procedure, purified by HPLC, and characterized by laser desorption mass spectrometry (Table 1). Prior to performing photo-inactivation reactions with recombinant His_6-carboxylase, we first determined the kinetic parameters of these properties. In the following studies, we used the concentrated, stable bovine ammonium sulfate-precipitated microsome carboxylase isolated from liver, because the kinetic properties of this form of carboxylase are well established and because purification of the bovine enzyme to near-homogeneity had no effect on the K_m and k_{cat} of the synthetic peptide substrate (Kuliopulos et al., 1992a; Morris et al., 1993). In addition, the recombinant His_6-carboxylase is kinetically indistinguishable from the wild-type bovine carboxylase (Kuliopulos et al., 1994).

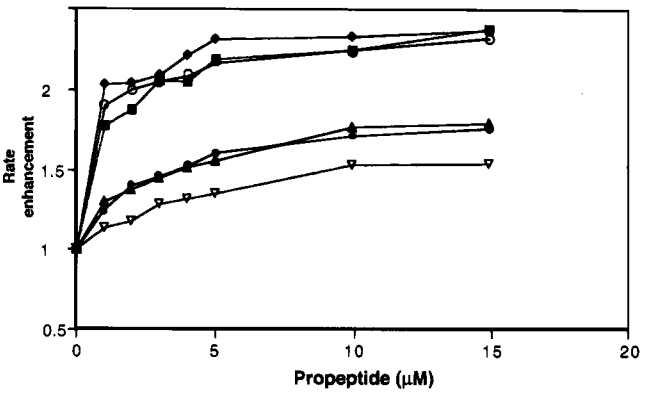


FIGURE 2: Rate enhancement of Bpa propeptides of FLEEL carboxylation. Carboxylase activity observed with 10 mM FLEEL in the presence of varying molar concentrations of the propeptides is shown. The reactions were at 25 °C in 125 μL systems containing 25 μL of ammonium sulfate-precipitated bovine liver microsomes [~380 ng of carboxylase in 2.7 mg of total protein with a specific activity of 3.2×10^5 dpm/h/(mg of total protein)$^{-1}$], 880 μM dihydrovitamin K_1, and 1.5 mM $NaH^{14}CO_3$ (10 μCi) in 150 mM NaCl, 20 mM sodium phosphate, and 0.8 M ammonium sulfate at pH 7.4 (Kuliopulos et al., 1992a). The reaction time was 6 min. The rate enhancements were performed with the following: proPT18 (■); proFIX19 (◆); proFIX19-16Bpa (▲); proFIX19-13Bpa (○); proFIX19-7Bpa (▽); proFIX19-6Bpa (●).

As shown in Figure 2 and Table 1, 10 μM proPT18 or proFIX19 enhanced γ-carboxylation of the synthetic FLEEL peptide by 2.2−2.3-fold, which is in close agreement with values obtained earlier (Knobloch & Suttie, 1987; Ulrich et al., 1988). The four Bpa peptides also enhanced γ-carboxylation by 1.5−2.3-fold, and their rate enhancement profiles were very similar to that of proFIX19, showing that these propeptides are recognized by the carboxylase.

The enhancement of carboxylation was further analyzed by K_m and V_m/K_m values. As shown in Table 1, this enhancement, brought on by propeptide, was the result of a lowering of the FLEEL K_m by about 5-fold, in agreement with earlier studies (Cheung et al., 1989, 1990). Also, the addition of 10 μM proPT18, proFIX19, or proFIX19-13Bpa enhanced the V_m/K_m values by about 5-fold. A moderately enhanced V_m/K_m of 2−3-fold was effected by proFIX19-16Bpa, proFIX19-7Bpa, and proFIX19-6Bpa. ProFIX19-13Bpa behaved comparably to proFIX19, possibly because residue −13 does not directly interact with carboxylase, as evidenced by the lack of conservation in residue −13 in factor IX, factor X, or prothrombin propeptides (Suttie, 1993).

Furthermore, the K_i values were determined for the effect of a Bpa propeptide on the carboxylation of the proPT28 substrate (residues −18 to +10 of prothrombin, which incorporates the complete propeptide and 10 residues of the mature amino terminus of prothrombin). As shown in Figure 3, proFIX19-16Bpa was a potent competitive inhibitor of the carboxylation of proPT28 by carboxylase with a K_i value of 17.4 μM (Table 1), which is only 5-fold weaker than the K_i for proPT18 (Ulrich et al., 1988). Together, these results

Table 1: Physical and Kinetic Parameters of Factor IX Bpa Propeptides

propeptide	molecular ion[a] (*m/z*)	FLEEL carboxylation K_m[b] (mM)	V_m[b] (dpm/min)	V_m/K_m[b] [M^{-1} (dpm/min)]	rate enhancement[c]	K_i[d] (μM)	% photo-inactivation[e]
none		9.0 ± 2.0	21 000	2.5×10^6			
proPT18		1.5 ± 0.2	30 000	2.0×10^7	2.2	3.5[f]	
proFIX19		1.6 ± 0.1	33 000	2.1×10^7	2.3		
proFIX19-16BPA	2430	1.9 ± 0.2	12 000	6.3×10^6	1.8	17.4	85
proFIX19-13BPA	2443	2.0 ± 02	37 000	1.9×10^7	2.2		43
proFIX19-7BPA	2464	2.0 ± 0.2	11 000	5.5×10^6	1.5		61
proFIX19-6BPA	2464	1.8 ± 0.2	13 000	7.2×10^6	1.7		51

[a] Determined by laser desorption mass spectroscopy. [b] Incorporation of $^{14}CO_2$ into the FLEEL peptide was linear up to 10 min when the reactions were done at 25 °C in 125 μL systems containing 25 μL of ammonium sulfate-precipitated bovine liver microsomes [~380 ng of carboxylase in 2.7 mg of total protein with a specific activity of 3.2×10^5 dpm/h/(mg of total protein)$^{-1}$], 880 μM dihydrovitamin K_1, and 1.5 mM $NaH^{14}CO_3$ (10 μCi) in 150 mM NaCl, 20 mM sodium phosphate, 0.8 M ammonium sulfate (pH 7.4), and 10 μM propeptide. Kinetic parameters for FLEEL carboxylation, K_m and V_m, were determined from double reciprocal plots of velocity with respect to substrate concentration by the hyperbolic weighted least-squares method. [c] Rate enhancement of the incorporation of $^{14}CO_2$ into the FLEEL peptide in the presence of 10 μM propeptide compared to the reaction carried out in the absence of propeptide. [d] K_i values were obtained by competitive inhibition experiments using proPT28 substrate. [e] The remaining carboxylase activities using the 59 amino acid residue substrate FIXQS were determined after photoaffinity inactivation for 15 min (see the text). [f] Ulrich et al., 1988.

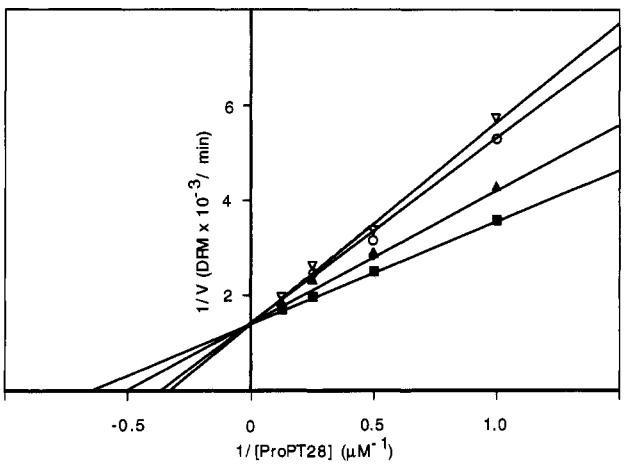


FIGURE 3: Inhibition of carboxylation of the proPT28 substrate by proFIX19-16Bpa. The initial velocities of carboxylation of proPT28 at saturating dihydrovitamin K_1 and $NaH^{14}CO_3$ were measured using carboxylase from bovine liver microsomes. These experiments were carried out under standard conditions (Kuliopulos et al., 1992a), except that 0.8 M ammonium sulfate was used as described in the text. The concentrations of proFIX19-16Bpa were 0 (■), 8 (▲), 12 (○), 16 (▽) μM. A K_i of 17.4 μM was determined.

argue that the four Bpa-containing factor IX propeptides retain high binding affinity at the carboxylase propeptide binding site, setting the stage for photoaffinity inactivation studies of the enzyme.

Photoinactivation of Carboxylase with Bpa Propeptides. For photoaffinity inactivation of carboxylase, 40 μM Bpa peptide (about 2-fold molar excess over K_i of proFIX19-16Bpa) was combined with bovine microsomal carboxylase, and the mixture was irradiated at 350 nm on ice (Shoelson et al., 1993; Williams & Shoelson, 1993). Estimates of the degree of inactivation were made by determination of the incorporation of $^{14}CO_2$ into FIXQS (−18 to 41), which includes the propeptide and all of the carboxylatable glutamic acid residues of the Gla domain of profactor IX (Wu et al., 1990). As shown in Table 1, in the presence of proFIX19-16Bpa, 85% of carboxylase activity was lost after 15 min of photoirradiation. Carboxylase underwent ≤15% activity loss in the absence of photoirradiation. With proFIX19-13Bpa, proFIX19-7Bpa, and proFIX19-6Bpa, 61−43% of activity was lost upon irradiation. ProFIX19-13BPA was the poorest inactivator (43%), which agrees with speculation that residue −13 is not oriented toward the molecular surface of carboxylase. In sum, all four Bpa propeptides, especially proFIX19-16BPa, at this point were candidates for specific ^{125}I labeling and mapping of carboxylase.

Specific and Irreversible Labeling of His_6-Carboxylase with [^{125}I]Tyrosyl Bpa Propeptides. For simultaneous inactivation and ^{125}I affinity labeling of carboxylase by the photo-cross-linking method, we used recombinant His_6-carboxylase since it is easily purified by its His_6 tag (Kuliopulos et al., 1994). Four [^{125}I]tyrosyl-proFIX19-Bpa-containing peptides were synthesized and purified by HPLC. The yields were 53−66%, and the final specific activities were approximately 2.1 Ci/μmol, which is sufficiently high to measureably label nanogram amounts of carboxylase. Photo-crosslinking reactions with [^{125}I]tyrosyl-labeled propeptides were carried out in stable microsome forms (Kuliopulos et al., 1994).

The His_6-carboxylase was incubated with 20 μCi of proFIX19-16Bpa[^{125}I]Y and photo-cross-linked under the same conditions as the bovine ammonium sulfate-precipitated enzyme described earlier. The inactivation mixtures were heated at 80 °C for 10 min in a dithiothreitol/SDS-containing sample buffer and analyzed by 10% SDS−PAGE. The gels were silver-stained, and radioactivity was assayed by autoradiography. As shown in Figure 4B, lane A, the His_6-carboxylase−proFIX19-16Bpa[^{125}I]Y cross-link material in microsomes could easily be identified as the 94 kDa carboxylase. Since the cross-linked complex persisted under conditions of 80 °C and electrophoresis, we concluded that the cross-linking interaction is irreversible and covalent.

After photo-cross-linking, the reaction mixture was applied to a 4 mL Ni−IDA affinity column under denaturating conditions (Kuliopulos et al., 1994). Bound His_6-carboxylase−proFIX19-16Bpa[^{125}I]Y was washed extensively with 5 mM imidazole-containing buffer to remove excess [^{125}I]-Bpa peptide and then eluted with 100 mM imidazole as shown in Figure 4A,B, lane B. The purity of the labeled 94 kDa His_6-carboxylase was about 10%, as quantitated by silver-stained SDS−PAGE gels, but comprised ≥80% of the ^{125}I-labeled material (Figure 4B, lane B), showing that it was about 2-fold purer than *N*-(bromoacetyl)-FLEEL[^{125}I]Y-labeled His_6-carboxylase from our previous studies (Kuliopulos et al., 1994). This result reinforces the idea that proFIX19-16Bpa[^{125}I]Y is a more specific inactivation reagent than *N*-(bromoacetyl)-FLEEL[^{125}I]Y (e.g., the K_m of

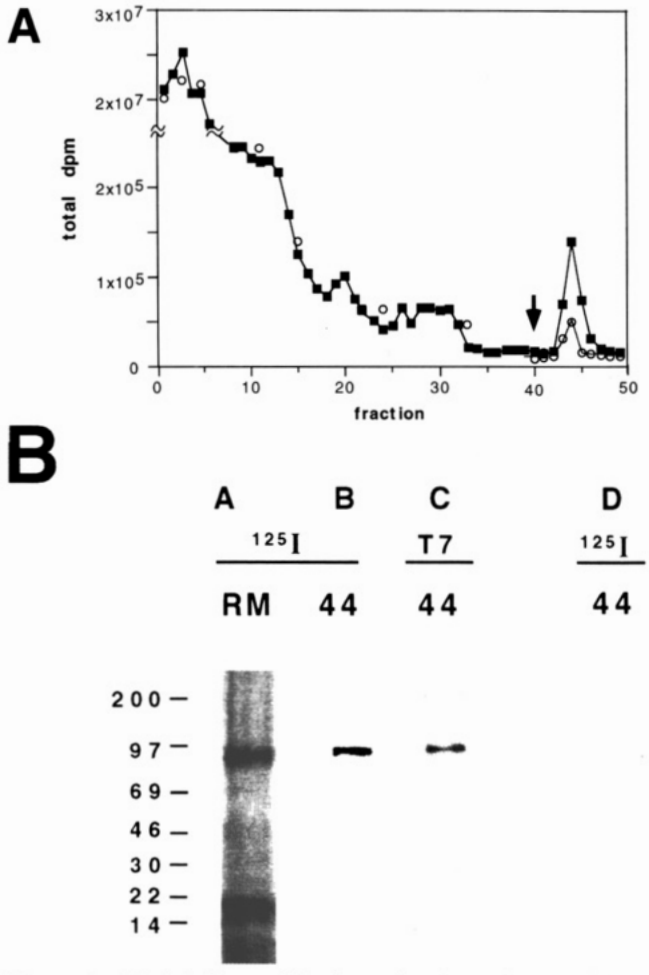


FIGURE 4: Ni chelation purification under denaturing conditions of His_6-carboxylase covalently labeled with proFIX19-16Bpa[^{125}I]Y. (A) Chromatograph of Ni−IDA purification of His_6-carboxylase−proFIX19-16Bpa[^{125}I]Y in the presence of 6.5 M urea. Each 1.5 mL column fraction was monitored for ^{125}I radioactivity as plotted on the ordinate (dpm). The vertical arrow represents a stepwise elution with buffer containing 100 mM imidazole. The His_6-carboxylase−proFIX19-16Bpa[^{125}I]Y was obtained by inactivation with 20 μCi of proFIX19-16Bpa[^{125}I]Y by the method described in the text (■) and by inactivation by the same method except that 5 μM proFIX19 was included (○) during inactivation. (B) 10% SDS−PAGE of desalted fractions from the Ni−IDA chromatograph shown in part A. Lanes A and B: Autoradiogram of inactivation reaction mixture (lane A) obtained by the inactivation of carboxylase with 20 μCi of proFIX19-16Bpa[^{125}I]Y and fraction 44 (lane B) (100 mM imidazole peak) from part A, respectively. Lane C: Western blot of lane B using the T7 tag antibody which recognizes residues −23 to −13 of His_6-carboxylase. Lane D: Autoradiogram of the 100 mM elution of a cross-linked inactivation reaction mixture, produced by the same method except that 5 μM proFIX19 was included during inactivation. Molecular masses in kilodaltons are shown on the left.

N-(bromoacetyl)-FLEELY is 800 μM and the K_i of proFIX19-16Bpa is 17 μM). The recovery of the labeled enzyme improved by 50% when we used a 4 mL Ni−IDA column as opposed to 1 mL column (about 30%), and the yield was 0.62% based on the starting radioactivity of proFIX19-16Bpa[^{125}I]Y. Western blots of the 100 mM imidazole-eluted material using the T7 antibody probe (fraction 44, Figure 4B, lane C) also identified the same full-length 94 kDa band that was labeled with proFIX19-16Bpa-[^{125}I]Y, confirming that this protein was the recombinant His_6-tagged carboxylase.

Addition of 5 μM proFIX19 propeptide to the photoactivation mixture substantially protected the carboxylase against labeling (Figure 4A, B, lane D). This result further supports the idea that inactivation occurs at the carboxylation propeptide recognition sequence, γ-CRS, of carboxylase.

FIGURE 5: 10% SDS−PAGE of desalted fractions from the Ni−IDA purification under denaturating conditions of His_6-carboxylase covalently labeled with Bpa peptides. Lanes A−D: Autoradiograms of Ni−IDA affinity-purified photo-inactivated His_6-carboxylase obtained by inactivation with 20 μCi of proFIX19-16Bpa-[^{125}I]Y, 20 μCi of proFIX19-13Bpa[^{125}I]Y, 20 μCi of proFIX19-7Bpa[^{125}I]Y, and 20 μCi of proFIX19-6Bpa[^{125}I]Y, respectively. Each lane contains approximately 20 ng of His_6-carboxylase. Molecular masses in kilodaltons are shown on the left.

Photo-inactivation reactions using proFIX19-13Bpa[^{125}I]Y, proFIX19-7Bpa[^{125}I]Y, and proFIX19-6Bpa[^{125}I]Y were carried out in the same way as with proFIX19-16Bpa[^{125}I]Y, and the resulting 100 mM imidazole buffer eluents from the Ni−IDA affinity column are shown in Figure 5. The 94 kDa band is the predominant labeled species, demonstrating that these labels were also very selective. The yields of His_6-carboxylase−proFIX19-13Bpa[^{125}I]Y, His_6-carboxylase−proFIX19-7Bpa[^{125}I]Y, and His_6-carboxylase−proFIX19-6Bpa[^{125}I]Y were 0.3−0.4%, which is about 50% less than that obtained with His_6-carboxylase−proFIX19-16Bpa[^{125}I]Y, in close agreement with the result obtained in the noniodinated experiment discussed earlier.

Proteolytic Digestions of Inactivated His_6-Carboxylase−ProFIX19-16Bpa[^{125}I]Y. We chose His_6-carboxylase−proFIX19-16Bpa[^{125}I]Y for proteolytic digestion and mapping studies due to its higher yield. The labeled enzyme was further purified away from the small quantity of remaining ^{125}I-labeled contaminants in the 100 mM imidazole-eluted fractions from the Ni−IDA affinity column by preparative gel electrophoresis (Kuliopulos et al., 1994). As shown in Figure 6, lane D, the 94 kDa His_6-carboxylase was substantially pure. Analysis of the Western blots of this purified protein using the T7-Ab, N-Ab, and C-Ab (Figure 6, lanes A, B, and C, respectively) probes followed by autoradiography confirmed that this protein was the labeled recombinant carboxylase. None of these bands were reactive with preimmune immunogloblin (data not shown). The T7 antibody, N-Ab, and C-Ab have previously been shown to react specifically with recombinant γ-carboxylase expressed in baculovirus-infected insect cells (Roth et al., 1993; Kuliopulos et al., 1994).

The purified His_6-carboxylase−proFIX19-16Bpa[^{125}I]Y was subjected to proteolytic digestion with endoproteinase Glu-C (*S. aureus* V8 protease, 5 μg for 1 h at room temperature) which had been previously shown to produce specific peptide fragments from carboxylase (Kuliopulos et

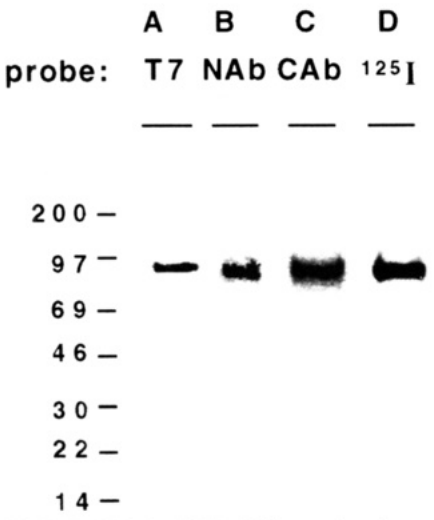


FIGURE 6: SDS–PAGE (4–20%) of His_6-carboxylase–proFIX19-16Bpa[^{125}I]Y purified by preparative polyacrylamide gel electrophoresis. Lanes A–C: Western blots of His_6-carboxylase–proFIX19-16Bpa[^{125}I]Y using T7-Ab, N-Ab, and C-Ab. Lane D: Autoradiogram of lane A. Molecular masses in kilodaltons are shown on the left.

al., 1994). Polypeptide fragments were resolved on 4–20% one-dimensional gradient SDS–PAGE gels and analyzed by Western blot with antibodies specific for discrete regions of the enzyme (T7-Ab, residues −23 to −13; N-Ab, residues 86–99; C-Ab, residues 661–673) and by autoradiography. As shown in Figure 7, lanes A and B, the protease released N-terminal proteolytic fragments that retained both the T7 tag (residues −23 to −13) and the ^{125}I affinity peptide label. These N-terminal fragments had relative masses of 57 kDa, corresponding to a cleavage at E462, or less likely at D468 or D475, because the V8 protease cleaves at Glu residues preferentially. The V8 protease digestion of the labeled recombinant carboxylase was also probed with C-Ab (Figure 7, lane C). The largest V8 protease fragment, which lost the ^{125}I label but retained the C-Ab epitope (residues 661–673), corresponds to a C-terminal 191 amino acid fragment (22 kDa) released by a single cleavage probably at E567, as shown in Figure 8. Thus, the 1 h digestion data indicate that the point of attachment of the propeptide affinity label is located in the first 462 amino acid residues of carboxylase.

More detailed information about the location of the photolabel was obtained by more extensive digestion experiments using 3 μg of V8 protease and overnight incubation. As shown in Figure 7, lanes D, F, and H, the N-terminal proteolytic fragments that were recognized by T7-Ab had an intact epitope for N-Ab (residues 86–99) and contained the ^{125}I peptide affinity label. These polypeptides had relative molecular masses of 57, 40, 33, and 30 kDa. The 57 kDa band is the same as that produced under 1 h digestion conditions, and the 30 kDa N-terminal fragment corresponds to a single cleavage, most likely at E225, which indicates that the propeptide binding/labeling site is located within the first 259 amino acid residues of the His_6-carboxylase including the 34 amino acid extension of the fusion protein (see Figure 8). The 20 kDa fragment, which maintained the ^{125}I label and the N-Ab epitope but lost the T7 tag (see Figure

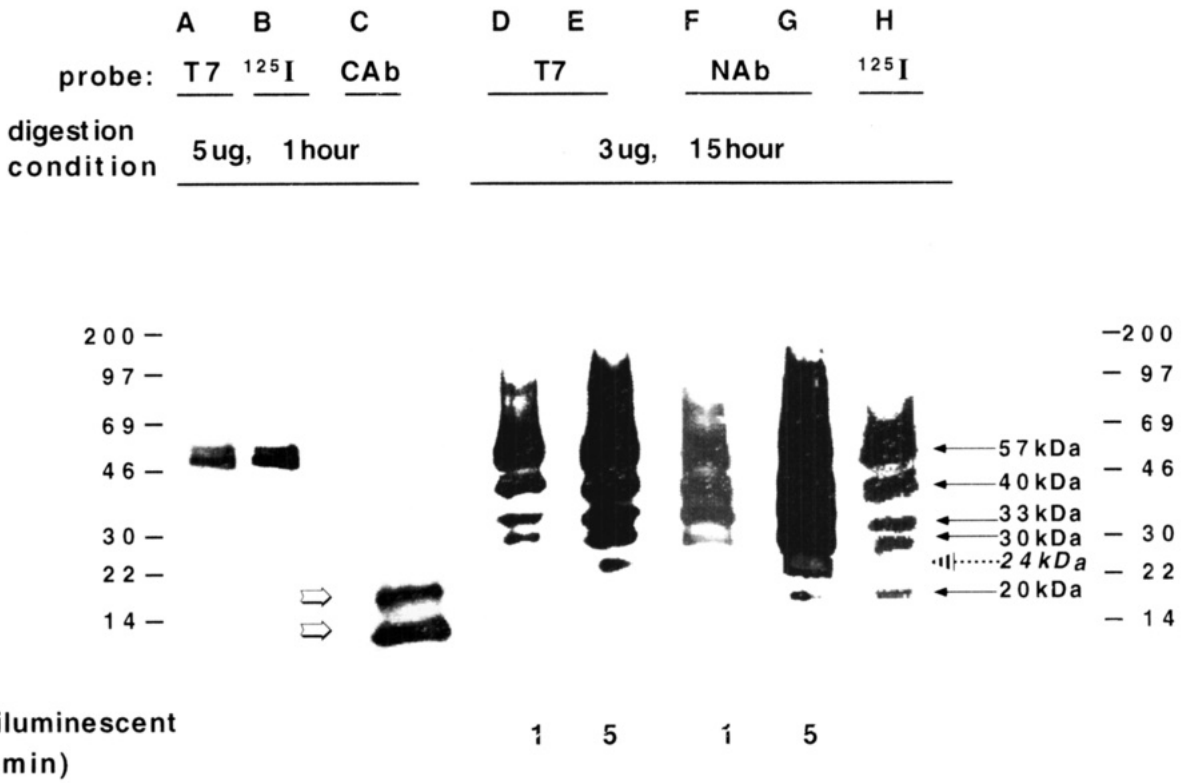


FIGURE 7: V8 protease digestions of His_6-carboxylase–proFIX19-16Bpa[^{125}I]Y. Lane A: Western blot using T7 antibody of His_6-carboxylase–proFIX19-16Bpa[^{125}I]Y digested for 1 h with 5 μg of endoproteinase Glu-C (V8) and exposed to X-ray film for 1 min. Lane B: Autoradiogram of lane A. Lane C: Western blot using C-Ab after a 1 h digestion with 5 μg of endoproteinase Glu-C. The open arrows indicate the bands detected by C-Ab that have lost the radiolabel. Lanes D and E: Western blots using T7-Ab on labeled carboxylase digested for 15 h with 3 μg of endoproteinase Glu-C (V8) and exposed to X-ray film for 1 and 5 min, respectively. Lanes F and G: Western blots using N-Ab on enzyme digested for 15 h with 3 μg of endoproteinase Glu-C (V8) and exposed to X-ray film for 1 and 5 min, respectively. Lane H: Autoradiogram of lanes D and E. Molecular masses in kilodaltons are shown on the extreme right and left sides. The five solid arrows indicate V8 proteolytic fragments of relative molecular masses 57, 40, 33, 30, and 20 kDa that contain the ^{125}I affinity label. The dotted arrow indicates the 24 kDa band detected by N-Ab, which lost the radiolabel.

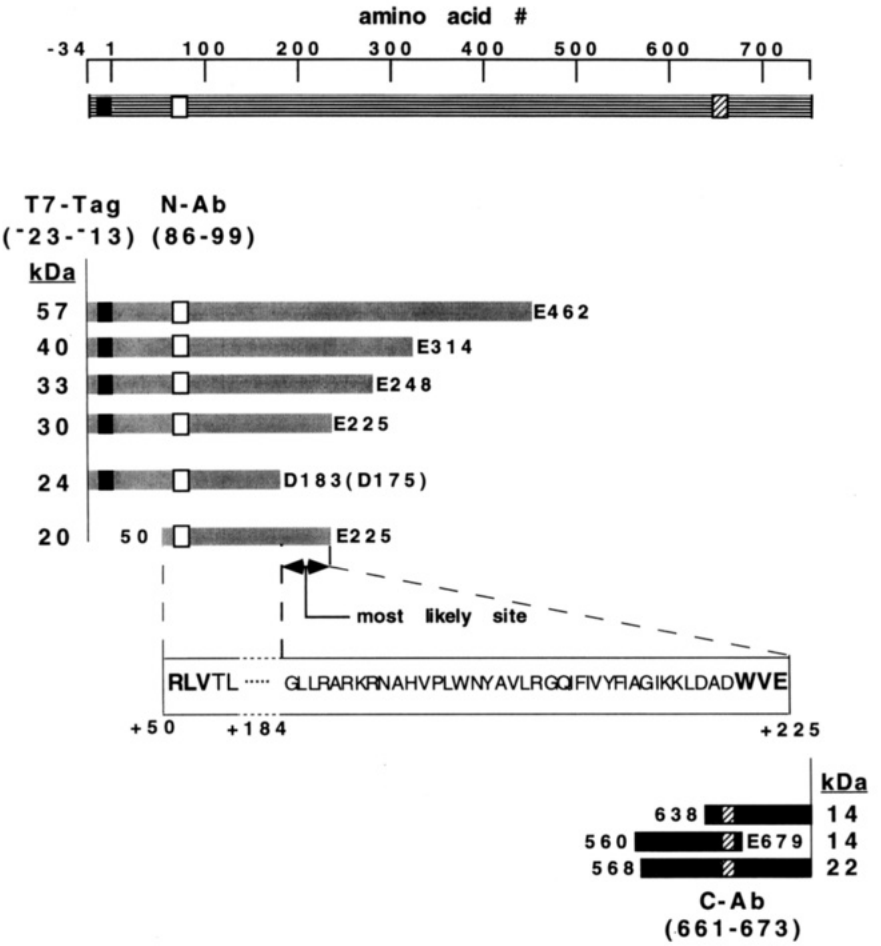


FIGURE 8: V8 proteolytic fragments of His_6-carboxylase and the resultant sequence of amino acids. The long, striped rectangle on top represents full-length His_6-carboxylase, with epitopes recognized by T7-Ab (black box), N-Ab (white box), and C-Ab (cross-hatched box). The gray rectangles represent sequential N-terminal V8 protease fragments generated by cleavage after Glu or Asp residues. V8 proteolytic fragments of relative molecular masses 57, 40, 33, and 30 kDa contain both proFIX19-16Bpa[^{125}I]Y and the T7 tag. The 24 kDa band that was detected by both N-Ab and T7-Ab lost the radiolabel, and the 20 kDa band that was detected by N-Ab contains the ^{125}I affinity label. The most likely site of propeptide attachment is indicated by the double-headed arrow. The sequence of amino acid residues in the 41-residue stretch from 184 to 225 is shown in the large box. The black rectangles at the bottom right of the figure represent limiting C-terminal V8 proteolytic fragments that retained the C-Ab epitope but lost the ^{125}I affinity label.

7, lanes E, G, and H), corresponds to a double cleavage at E49 and E225, producing a polypeptide of 198 amino acid residues. Together, these results demonstrate that the propeptide binding/activation site on carboxylase is localized to a hydrophobic region comprising residues Arg +50 to Glu +225 (see Figure 8). This is consistent with our previous studies using the *N*-(bromoacetyl)-FLEEL[^{125}I]Y carboxylase substrate affinity label (see Figure 9), indicating that the ^{125}I label is located within the first 218 amino acid residues of the 758-residue carboxylase (Kuliopulos et al., 1994), because the propeptide recognition site and the active site of the enzyme are expected to be adjacent (Furie & Furie, 1990).

Importantly, V8 digestion of the His_6-carboxylase yielded a 24 kDa band, which retained both the T7 tag and the N-Ab epitope but lost the ^{125}I label (see Figure 7, lanes E, G, and H). This fragment could be generated by cleavage at D175 or D183. This result shows that the propeptide label is most likely located within the region stretching from Gly +184 to Glu +225, e.g., a 41 amino acid residue stretch of the 758 amino acid full-length carboxylase (see Figure 8).

Proteolytic Digestions of Inactivated His_6-Carboxylase−ProFIX19-13Bpa[^{125}I]Y and His_6-Carboxylase−ProFIX19-7Bpa[^{125}I]Y. In parallel, labeled His_6-carboxylase−proFIX19-13Bpa[^{125}I]Y and His_6-carboxylase−proFIX19-7Bpa[^{125}I]Y were purified by preparative gel electrophoresis and digested with V8 protease. As shown in Figure 10, endoproteinase V8 released discrete N-terminal fragments with relative

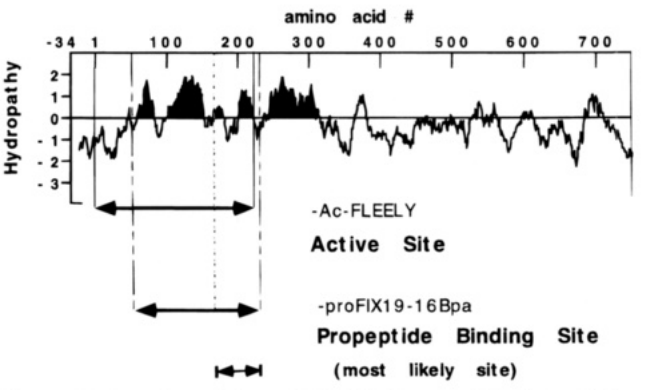


FIGURE 9: Location of the proFIX19-16Bpa peptide photoaffinity inactivation site and the *N*-(bromoacetyl)-FLEELY peptide inactivation site on carboxylase. Kyte−Doolittle hydropathy analysis of the carboxylase sequence using a sliding window of 19 amino acids is shown on top. The N-terminal hydrophobic region shown in black is flanked by a short N-terminal hydrophilic region and a much longer C-terminal hydrophilic region. The overlapping regions labeled by either the active site probe, Ac-FLEELY, or the proFIX19-Bpa propeptide probe are indicated by double-headed arrows. The 41 amino acid region from 184 to 225 is indicated as the most likely propeptide site of attachment.

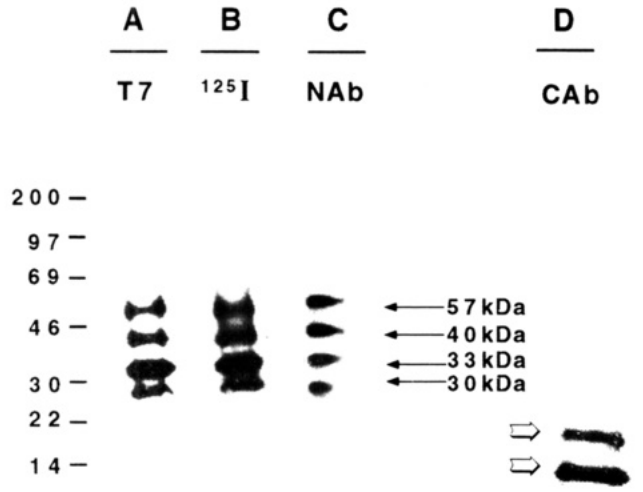


FIGURE 10: V8 protease digestions of His_6-carboxylase−proFIX19-13Bpa[^{125}I]Y. Lane A: Western blot using the T7 antibody of His_6-carboxylase−proFIX19-13Bpa[^{125}I]Y digested for 15 h with 3 μg of endoproteinase Glu-C (V8) and exposed to X-ray film for 1 min. Lane B: Autoradiogram of lane A. Lane C: Western blot using N-Ab of labeled enzyme digested for 15 h with 3 μg of endoproteinase Glu-C (V8) and exposed to X-ray film for 1 min. Molecular masses in kilodaltons are shown on the left. The four arrows indicate V8 proteolytic fragments of relative molecular masses 57, 40, 33, and 30 kDa that contain the ^{125}I affinity label. Lane D: Western blot using C-Ab on enzyme digested for 15 h with 3 μg of endoproteinase Glu-C. The open arrows indicate the bands detected by C-Ab, which lost the radiolabel.

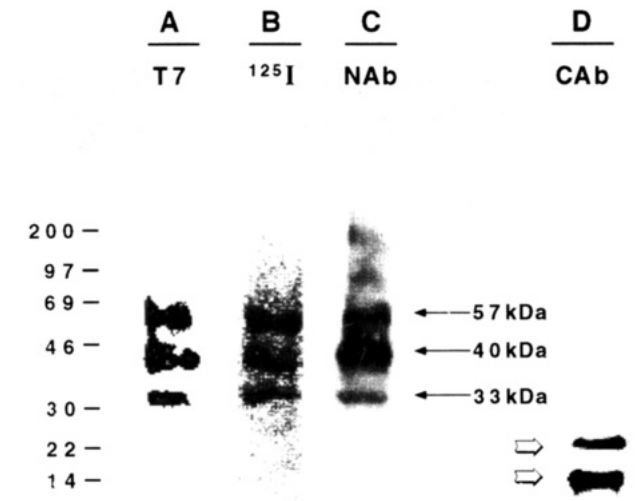


FIGURE 11: V8 protease digestions of His_6-carboxylase−proFIX19-7Bpa[^{125}I]Y. Lane A: Western blot using T7 antibody of His_6-carboxylase−proFIX19-7Bpa[^{125}I]Y digested for 15 h with 3 μg of endoproteinase Glu-C (V8) and exposed to X-ray film for 1 min. Lane B: Autoradiogram of lane A. Lane C: Western blot using N-Ab of labeled enzyme digested for 15 h with 3 μg of endoproteinase Glu-C (V8) and exposed to X-ray film for 1 min. Molecular massess in kilodaltons are shown on the left. The three arrows indicate V8 proteolytic fragments of relative molecular masses 57, 40, and 33 kDa that contain the ^{125}I affinity label. Lane D: Western blot using C-Ab on enzyme digested for 15 h with 3 μg of endoproteinase Glu-C. The open arrows indicate the bands detected by C-Ab, which lost the radiolabel.

masses of 57, 40, 33, and 30 kDa from His_6-carboxylase−proFIX19-13Bpa[^{125}I]Y, which maintained the N-Ab epitope, T7 tag, and ^{125}I label. This result shows that the proFIX19-13Bpa propeptide is also localized in the amino-terminal one-third of the carboxylase (30 kDa).

As shown in Figure 11, the V8 protease also produced a series of 57, 40, and 33 kDa bands from His_6-carboxylase−proFIX19-7Bpa[^{125}I]Y, which had an intact N-Ab epitope, T7 tag, and ^{125}I label. These results are coincident with the *N*-(bromoacetyl)-FLEELY and proFIX19-16Bpa labeling results, independently confirming for the third time that the propeptide binding site is located in the first one-third of the hydrophobic amino terminus of carboxylase. Similar results were obtained with V8 protease-digested His_6-carboxylase−proFIX19-6Bpa[^{125}I]Y (data not shown). V8 protease produced both the 22 and 14 kDa bands with high reproducibility by cleavage at E567 of His_6-carboxylase−proFIX19-13Bpa[^{125}I]Y and His_6-carboxylase−proFIX19-7Bpa[^{125}I]Y, as detected by the C-Ab (Figure 10, lane D, and Figure 11, lane D), ruling out nonspecific labeling of the C-terminal 191−345 amino acid residues of carboxylase.

DISCUSSION

Specificity of Bpa Propeptides. The Bpa residue has an aryl ketone moiety, which is chemically stable, conveniently manipulated in ambient light, photolyzable on 350 nm UV light irradiation, and selectively active with minimal damage to the protein. In addition, it preferentially attacks weak C−H bonds in the local environment with high selectivity and yield (Dormán & Prestwich, 1994). Also, the benzophenone moiety should directly attach to any amino acid residue of carboxylase without requirement for a nucleophilic side chain on the enzyme. Thus, we have prepared and tested variants of the factor IX propeptide-containing Bpa substituents at four sites spanning residues −16 to −6. All four proFIX19-Bpa regioisomers bind to and activate carboxylase for CO_2 incorporation into FLEEL, suggesting that the Bpa side chains do not substantially disrupt propeptide−carboxylase recognition. Moreover, all four Bpa propeptides (−16, −13, −7, and −6) cause photo-inactivation of carboxylase, and all four ^{125}I-labeled Bpa propeptides specifically attach to the 94 kDa carboxylase. The fact that all four Bpa peptide regioisomers covalently derivatize the same amino-terminal proteolytic fragments of carboxylase provides four parallel indications that this is indeed the γ-carboxylase propeptide recognition site (γ-CRS). Although sufficient quantities of His_6-carboxylase were not yet able to be expressed in our pBlueBacHisB baculovirus transfer vector system to obtain sequencing data (2 μg of carboxylase/500 μL of solublized microsome; Kuliopulos et al., 1994), if enough carboxylase can be obtained subsequently to identify the particular residues labeled by the four Bpa propeptides, this should constitute a photoaffinity scanning procedure (Shoelson et al., 1993) to map the −16 to −6 interface of the propeptide to the carboxylase γ-CRS. That the γ-CRS site and the site labeled by the substrate/inactivator *N*-(bromoacetyl)-FLEELY are both in the first one-third of the 758 amino acid carboxylase is also consistent with the close apposition of propeptide (−18 to −1) and carboxylatable glutamyl residues (+6 to ca. +40) in protein substrates. The 41-residue stretch of enzyme from Gly 184 to Glu 225 is now a reasonable target for mutagenesis studies to probe the recognition of both propeptide and glutamyl peptide substrates (Sugiura et al., 1994).

The Bpa propeptides afford a direct analysis of the propeptide−carboxylase interface in the γ-CRS region of the enzyme, without the need for a separate cross-linking reagent. Such cross-linkers have been used previously by Wu et al. (1991b) to attach ^{125}I-labeled FIXQS to carboxylase, but no sublocalization results were described. Most recently, Wu et al. (1994) reported cross-linking of propeptide to the

C-terminal region of carboxylase, but the nature of the propeptide and cross-linkers used has not been detailed.

Given the initial mapping of propeptide and substrate binding sites on carboxylase, the recognition region for vitamin KH_2 remains to be defined. In view of the lipid-soluble nature of KH_2 one might expect binding to a hydrophobic region of carboxylase, but the existence of two soluble, low molecular weight hydroquinone microbial epoxidases (Shen & Gould, 1991) may indicate a role for a putative hydrophilic C-terminal domain of carboxylase.

Importance of the Hydrophobic Membrane Region of Carboxylase. Indication that propeptide and substrate recognition may be in the predicted hydrophobic region (Figure 9) of the amino-terminal portion of carboxylase suggests that a hydrophobic microenvironment may be important for the unusual catalytic abilities of this enzyme (Suttie et al., 1993; Kuliopulos et al., 1994). Both model and enzymatic studies suggest that a dioxygenated intermediate formed from vitamin KH_2, which is thought to have a pK_a of approximately 20, acts as a base for abstraction of the γ-methylene hydrogen from carboxylable Glu residues (Dowd et al., 1991, 1992; Kuliopulos et al., 1992b). However, it is still difficult to understand how this alkoxide epoxy K is strong enough to pull off the γ-Glu proton with an estimated pK_a value of 26−28 (Gronert & Streitwieser, 1988; Renaud & Fox, 1988). We propose that a hydrophobic active site region of carboxylase would lower the pK_a of the γ-proton on the Glu residues, if the carbanion intermediate resulting from proton abstraction is stabilized in the hydrophobic, bulk solvent-excluded environment of the enzyme active site, possibly using a concerted enolization mechanism to reduce high-energy transition state barriers. This has been proposed in the formation of strong hydrogen bonds between the resultant anionic conjugate base and enzyme side chains (Gerlt & Gassman, 1993; Cleland & Kreevoy, 1994).

Taken together, we assume that both the strong base generated from oxygenation of coenzyme KH_2 and the hydrophobic reaction site environment may contribute to the base abstraction mechanism of carboxylase and would impart a mechanistic rationale to the architecture and location of the active site of this enzyme.

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