# Multi-Site-Specificity of the Vitamin K-Dependent Carboxylase: *In Vitro* Carboxylation of Des-$\gamma$-carboxylated Bone Gla Protein and Des-$\gamma$-carboxylated Pro Bone Gla Protein[†]

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ABSTRACT: The vitamin K-dependent carboxylase processes multiple glutamic acid residues to $\gamma$-carboxyglutamic acid (Gla) residues in a limited number of proteins. The targeted proteins are synthesized with an amino-terminal propeptide which has been shown to play an important role in $\gamma$-carboxylation. The specificity of the enzyme for each potential Gla site, the direction of carboxylation, and the influence of a bound propeptide on these events are not understood. Des-$\gamma$-carboxy forms of bone Gla protein (BGP), which contain potential Gla residues at positions 17, 21, and 24, were employed as model substrates to determine the multi-site-specificity of the enzyme. Recombinant bovine des-$\gamma$-carboxylated proBGP (rdproBGP) and heat-decarboxylated BGP (dBGP), lacking a propeptide, were used as substrates for a bovine liver carboxylase, and the *in vitro* reaction products were analyzed for the formation of $^{14}CO_2$ Gla. The di-Gla species was found to be the predominant product of *in vitro* carboxylation of both rdproBGP and dBGP at less than saturating concentrations of each substrate. Carboxylation of both substrates occurred preferentially at the more C-terminal potential Gla sites, residues 21 and 24. A similar pattern of carboxylation was observed with a rat bone cell carboxylase, suggesting no species or tissue variation in the enzyme specificity. Some tricarboxylated product accumulated during carboxylation of rdproBGP but not dBGP, suggesting that the covalently bound propeptide directs more complete carboxylation of the Gla domain. In addition, monocarboxylated rdproBGP was found to accumulate in the absence but not in the presence of a free noncovalently attached propeptide, indicating that free propeptide affects more efficient carboxylation of rdproBGP.

The vitamin K-dependent carboxylase, located at the luminal surface of the rough endoplasmic reticulum, catalyzes the posttranslational conversion of glutamic acid residues to $\gamma$-carboxyglutamic acid (Gla) residues in a limited number of proteins. These proteins include the plasma clotting factors II (prothrombin), VII, IX, and X and the plasma proteins C, S, and Z as well as bone Gla protein (BGP), also called osteocalcin, and matrix Gla protein (MGP). Multiple Glu residues within these substrates are targeted by the enzyme for carboxylation. Precursors of the vitamin K-dependent proteins contain an N-terminal propeptide, and it is clear from both *in vivo* and *in vitro* studies that this pro region plays a role as a recognition site for the carboxylase (Suttie, 1993); however, the multi-site-specificity and directionality of the enzyme are not understood.

Liska and Suttie (1988) investigated the location of Gla residues in preparations of the partially $\gamma$-carboxylated pool of prothrombin isolated from the plasma of a cow rendered vitamin K-deficient by administration of a 4-hydroxycoumarin anticoagulant. In all of the variants analyzed, the most N-terminal potential Gla site was the most completely carboxylated. The extent of carboxylation of the other nine residues differed between variants, and a defined order of carboxylation could not be determined. In a similar study, Borowski *et al.* (1986) found the most amino-terminal potential Gla site in under-$\gamma$-carboxylated human prothrombin genetic variants to be fully carboxylated. The extent of carboxylation at the other nine sites differed between variants and also differed significantly from the pattern of carboxylation seen in the Liska and Suttie (1988) study. *In vitro* studies of Gla formation have been limited to the use of short, synthetic peptide substrates containing Glu-Glu sequences. Decottignies-Le Marechal *et al.* (1979) and Finnan and Suttie (1980) analyzed the extent of carboxylation of the carboxylase substrate Phe-Leu-Glu-Glu-Leu and found that only the first Glu residue was appreciably carboxylated. These data would suggest that the specificity of the carboxylase is highest for the most N-terminal Glu site, but do not clearly establish whether carboxylation is a random or an ordered event. A direct assessment of the specificity or directionality of *in vitro* Gla formation in a vitamin K-dependent protein substrate has not been reported.

To address the multi-site-specificity of the $\gamma$-glutamyl carboxylase, a protein which was a physiological substrate for the enzyme yet contained few potential Gla residues so as to simplify interpretation of the results was desired.

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Bovine bone Gla protein met these criteria and was chosen as a model substrate. Bovine BGP is a 49-residue protein which contains 3 Gla residues located at positions 17, 21, and 24 (Price *et al.*, 1976). Bovine BGP was isolated from bone and heated to decarboxylate Gla residues to Glu residues, and the resulting des-$\gamma$-carboxy BGP (dBGP) was used as a substrate for a bovine liver vitamin K-dependent $\gamma$-glutamyl carboxylase preparation. Following incubation with the bovine liver carboxylase, the products were analyzed to determine the incorporation of $^{14}CO_2$ into the three potential Gla sites. A recombinant bovine des-$\gamma$-carboxylated BGP precursor (rdproBGP) was expressed in baculovirus-infected insect cells and was also utilized as a substrate for the carboxylase. The importance of the covalently bound propeptide on the extent and specificity of carboxylation was assessed by comparing the patterns of carboxylation of dBGP and rdproBGP.

## MATERIALS AND METHODS

*Des-$\gamma$-carboxy Bone Gla Protein (dBGP) Substrate.* Bovine BGP was prepared from calf cortical bone and heat-decarboxylated by modification of an earlier procedure of Poser and Price (1979). BGP (10−20 mg) was freed of divalent metal ions by filtration over a Sephadex G25 column equilibrated with 100 mM $NH_4HCO_3$ containing 0.5 mM of the ammonium salt of EDTA at pH 8. The protein was lyophilized to dryness and heated under vacuum for 6 h at 110 °C. To ensure complete decarboxylation, the protein was then dissolved in 2 mL of 50 mM $NH_4HCO_3$, lyophilized to dryness, and again heated under vacuum for 4 h at 110 °C.

*Des-$\gamma$-carboxy Pro Bone Gla Protein (dproBGP) Substrate. (A) Plasmids, Insect Cells, and Virus.* Bovine and human preproBGP cDNAs in Bluescript SK(−) plasmids were gifts from Chiron Corp. (Emeryville, CA). The baculovirus transfer vector pEVbsmer (Lerch & Friesen, 1992) was obtained from Dr. Paul Friesen (University of Wisconsin−Madison) with permission from the Genetics Institute, Inc. (Cambridge, MA). *Spodoptera frugiperda* cells (Sf21) were maintained in pH 6.2 TC100 growth medium supplemented with 10% heat-inactivated fetal bovine serum, 2.5 g/L tryptose broth, 0.06 g/L penicillin G, 0.27 g/L streptomycin sulfate, and 0.6 mg/L amphotericin B. The cells were grown in monolayer or in suspension in spinner flasks at 27 °C. The wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA (Lee & Miller, 1978) was a gift from Dr. Friesen.

*(B) Generation of Recombinant preproBGP Baculovirus.* Each preproBGP cDNA was subcloned from a Bluescript SK(−) plasmid into the pEVbsmer vector. The human and bovine cDNA initiation codons were 86 base pairs (bp) and 100 bp, respectively, from the 5′ polyhedron leader. Each transfer vector (10−15 $\mu$g) was linearized with *Nde*I and cotransfected with wild-type AcNPV DNA into Sf21 cells in the presence of lipofectin according to the manufacturer's specifications. Virus was harvested after a 96 h incubation at 27 °C, and recombinant virus was obtained following three rounds of purification of occlusion negative plaques with subsequent titering by the plaque assay (Summer & Smith, 1987).

*(C) Recombinant Expression and Purification of proBGP.* Sf21 cells were plated at $1 \times 10^7$ cells per 100 mm plate. Between 8 and 10 plates were infected at a multiplicity of infection (MOI) of 10 and harvested at 48 h postinfection (hpi). The cells were scraped off each plate, pooled, pelleted, and lysed as described by Harlow and Lane (1988) by resuspending in 1 mL per $10^7$ cells of Nonidet P-40 buffer (1% Nonidet P-40/0.05 M Tris-HCl/0.15 M NaCl/pH 8) containing 100 $\mu$g/mL phenylmethanesulfonyl fluoride, 100 $\mu$M benzamidine, 1.5 mM EDTA, and 2 $\mu$g/mL aprotinin. Following a 30 min incubation on ice, the lysate was centrifuged at 10000*g* for 10 min, the supernatant was centrifuged at 100000*g* for 30 min, and this supernatant was either frozen in liquid nitrogen or applied directly to a 1.5 mL anti-BGP antibody resin (5 mg/mL gel) prepared as described by Pharmacia Fine Chemicals (1983) and nutated 3 h at 4 °C. The resin was washed exhaustively with phosphate-buffered saline (PBS) followed by 0.2 M Tris-HCl/0.5 M NaCl/pH 7.5. The bound protein was eluted with 4 M guanidine hydrochloride and dialyzed against SIK buffer (0.25 M sucrose/0.5 M KCl/0.025 M imidazole). The bovine proBGP in crude cell lysate, in media, and in the antibody-purified preparation was quantitated with a BGP radioimmunoassay as previously described (Price & Nishimoto, 1980). Immunoaffinity-purified proBGP from solubilized cell lysate was subjected to 17.5% SDS/PAGE and transferred to an Immobilin-P membrane (Matsudaira, 1987). The immobilon-bound protein was visualized by Coomassie staining, the band was excised from the membrane, and N-terminal sequence analysis was carried out by the Protein Sequence Laboratory of the Medical College of Wisconsin (Milwaukee, WI).

*(D) Gel Electrophoresis and Immunoblot Analysis.* Media samples were prepared by replacing media with serum-free media at 24 hpi, harvesting at 48 hpi, and directly mixing and boiling equal volumes of media and sample buffer prior to loading. Cells were pelleted at 48 hpi and boiled 10 min in sample buffer, and the equivalent of $1 \times 10^5$ cells were loaded per lane. Samples were electrophoresed in 17.5% SDS/polyacrylamide gels (Laemmli, 1970), and transferred to Immobilon-P (Millipore Corp., Bedford, MA) membrane for immunoblot analysis. The membrane was incubated with antigen-purified rabbit polyclonal BGP antibodies. Following a second incubation with anti-rabbit IgG (Promega Corp., Madison, WI) bound to alkaline phosphatase, bands were visualized via color development upon addition of alkaline phosphatase substrates, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate (Promega Corp.).

*BGP Antibody Production and Purification.* White New Zealand male rabbits (Hazleton Research Products Inc., Denver, PA) were immunized with 1 mg of bovine BGP and boosted with 0.5 mg of BGP 3 weeks later, and high-antibody titers were maintained by boosting every 3 months with 0.5 mg of antigen. Inoculum was prepared by adsorbing the BGP to polyvinylpyrrolidone as described by Worobec *et al.* (1972), and emulsifying in either complete (initial challenge) or incomplete Freund's adjuvant. Blood was drawn on a weekly basis, and the serum was stored at −20 °C. Antiserum was purified by antigen adsorption essentially as described by Poser *et al.* (1980), and antibodies were coupled to CNBr-preactivated Sepharose 4B as described by Pharmacia (1983).

*Carboxylase Assays and Isolation of Carboxylated Products.* A crude bovine liver microsomal fraction was prepared and solubilized as previously described (Berkner *et al.*, 1992).

A 500−1000-fold purified preparation of the bovine liver vitamin K-dependent carboxylase, containing 100 $\mu$M human factor X (hFX) propeptide, was prepared as previously described (Harbeck *et al.*, 1989). Reaction mixtures contained 700 $\mu$L of enzyme preparation (20 mg of protein/mL of solubilized microsomes or 0.15 mg of protein/mL of the semipurified preparation) added to an equal volume of other reactants such that the final 1.4 mL volume contained dBGP or rdproBGP in SIK buffer, 50 $\mu$Ci/mL $NaH^{14}CO_3$, 150 $\mu$g/mL reduced vitamin K ($KH_2$), 0.6 M $(NH_4)_2SO_4$, and 10 mM DTT. An additional 50−100 $\mu$M hFX propeptide was added to some crude microsomal preparations. Reactions were carried out at 25 °C in a shaking waterbath for the times indicated and stopped by bubbling under a stream of $CO_2$ for 5 min.

The products and unreacted substrate were isolated on 4 mL of an anti-BGP antibody resin (2 mg/mL) by gravity filtration. The resin was washed with 2 column volumes of 0.2 M Tris-HCl/0.5 M NaCl/pH 7.5 and eluted with 4 M guanidine hydrochloride, and the denaturant was subsequently removed on an Econo-Pac 10DG desalting column (Bio-Rad) equilibrated in 50 mM $NH_4OH$. The isolated products were lyophilized to dryness and stored at −20 °C. When purified carboxylase was used as the enzyme source, 0.5 mL of solubilized bovine liver microsomes was added to the reaction mixture just prior to isolation on the antibody column to prevent $^{14}$C-rproBGP adsorption to the Econo-Pac column.

Assays of carboxylase activity in solubilized cell extracts were carried out according to a modification of the procedure described by de Boer-van den Berg *et al.* (1987). A 1 mL volume of thawed and solubilized cell extracts (2−3 mg/mL) was added to an equal volume of other reactants such that the final 2 mL volume contained 0.6 M ammonium sulfate, 30 $\mu$g of $KH_2$, 20 $\mu$Ci of $NaH^{14}CO_3$, 7 mM DTT, 25 $\mu$M hFX propeptide, and 0.8−0.9 $\mu$M rdproBGP. Reactions were carried out at 25 °C in a shaking waterbath for 3 h. The mixture was then centrifuged for 1 h at 10000*g* to remove cell debris, and the supernatant was subsequently bubbled under a stream of $CO_2$ for 5 min to remove unincorporated $^{14}CO_2$. Approximately 0.5 mL of solubilized bovine liver microsomes was added, and the products and remaining reactants were immediately isolated as described above for microsomal carboxylated rproBGP.

*Isoelectric Focusing Gel Electrophoresis.* Lyophilized protein was suspended in sample buffer (50 mM $Na_2HPO_4$/7.5 M urea/1% $\beta$-mercaptoethanol/pH 8) and incubated 15 min at 40 °C. Samples were applied to dry wells of 7.5% polyacrylamide gels containing 7 M urea/2% pharmalytes (pH 2.5−5 for BGP and pH 4−6.5 for rproBGP)/0.3 mg/mL ammonium persulfate/0.1% TEMED and overlayed with 50 mM imidazole/10% glycerol/pH 7. The top and bottom reservoirs were filled with 0.1 M NaOH and 0.1 M $H_2SO_4$, respectively. Gels were run 18−20 h at 250 V and then 60 min at 400 V. Protein was fixed 2 h in 10% trichloroacetic acid and stained with Coomassie brilliant blue. After being rinsed thoroughly with water, gels were incubated 30 min in Fluoro-Hance, dried on a gel dryer, and subjected to fluorography. Quantitation of individual bands was carried out by scanning gels with a Blot Analyzer (Betagen Corp.). Mono- and dicarboxylated BGP species were isolated from unstained IEF gels after they were located by the use of stained markers in adjacent lanes. Proteins were eluted into 600 mL of 0.3 M $NH_4HCO_3$/pH 8 per band at 37 °C for 5 h. Eluted bands were combined, the volume was adjusted to 3 mL with water, and 0.3 mg of BGP was added as a carrier. Samples were applied to an Econo-Pac 10DG desalting column (Bio-Rad), and the eluate, free of urea, was dried under a stream of filtered air.

*Identification of Carboxylated Glu Sites. (A) Heat-Decarboxylation of Gla Residues.* The general procedure of Poser and Price (1979) as modified above was followed. On the basis of isoelectric focusing gel electrophoresis of the final product, >90% of the Gla was converted to Glu.

*(B) Reduction and S-Carboxymethylation.* Approximately 1 mg of heat-decarboxylated BGP/$^{14}$C-BGP was reduced and carboxymethylated as described by Huq *et al.* (1987). The completed reaction mixture was transferred to dialysis tubing and dialyzed into 1% $NH_4HCO_3$/pH 8 at 4 °C.

*(C) Trypsin Digestion and Peptide Isolation.* Lyophilized $^{14}$C-BGP, or $^{14}$C-rproBGP isolated from carboxylase assays, was dissolved in 0.1 M $NH_4HCO_3$/pH 8 and 1 mg or 0.3 mg of BGP added as a carrier. These samples were digested with TPCK-treated trypsin in a 1:100 (w/w) ratio for 2 h at 25 °C. A second aliquot of trypsin was added, the reaction was contained for 14−15 h, and the digest was lyophilized to dryness. Reverse phase HPLC separations of the trypsin-digested carboxylase products were carried out using Waters (Milford, MA) system controllers and pumps and a Hamilton PRP-1 analytical column (15 × 4.1 mm, 10 $\mu$m) equilibrated in buffer A [2% acetonitrile/$H_2O$/0.1% trifluoroacetic acid (TFA)]. A linear gradient was developed upon injection from 0 to 100% buffer B (60% acetonitrile/$H_2O$/0.1% TFA) in 60 min with a flow rate of 1 mL/min. The eluant was monitored at 220 nm, and peptide fragments containing BGP residues 1−9 and 20−43 were collected and dried under a stream of filtered air.

*(D) Determination of the Extent of Gla Formation.* Tryptic fragments were suspended in 200 $\mu$L of 6 N constant-boiling HCl in Eppendorf tubes and placed in 18 × 150 mm glass tubes which were evacuated and sealed. After 22 h at 110 °C, the tubes were opened, the HCl was evaporated under a stream of filtered air, and the samples were resuspended in $H_2O$, aliquoted to 6 × 50 mm glass tubes, lyophilized to dryness, and stored at −20 °C. These acid hydrolysates were derivatized with 11 $\mu$L of coupling solution [200 $\mu$L of acetonitrile, pyridine (PYR), $H_2O$, and triethylamine (TEA) (10:5:3:2)] plus 20 $\mu$L of phenyl isothiocyanate (PITC) for 30 min at room temperature. The samples were dried under a stream of $N_2$ and stored at 4 °C prior to same day analysis.

Phenylthiocarbamyl-Glu (PTC-Glu) was quantitated after reverse phase HPLC separation as described by Bergman *et al.* (1986). Elution of the column was monitored at 254 nm, and PTC-Glu peaks were collected, dried under a stream of $N_2$, dissolved in 100 $\mu$L of $H_2O$, and subsequently dissolved in 0.7 mL of tissue solubilizer. The mixture was suspended in 3 mL of Bio-Safe II and transferred to a mini-scintillation vial, and $^{14}$C-PTC-Glu was quantitated in a liquid scintillation spectrometer. A standard curve of PTC-Glu was developed using Amino Acid Standards H (Pierce), derivatized, and detected as stated above. Linear regression analysis was carried out on the peak areas and nanomoles of PTC-Glu calculated. Specific activity was calculated as dpm per nanomole of PTC-Glu.

*(E) Manual Edman Sequencing.* Fragment 20−43 from the tryptic digestion of BGP was dissolved in $H_2O$, trans-

ferred to an Eppendorf tube, lyophilized to dryness, and coupled to half of a membrane supplied in the Sequelon AA Attachment Kit (Milligen/Biosearch, Burlington, MA) as described in the manual. The membranes were rinsed with 30% acetonitrile/$H_2O$ and transferred to 6 × 50 mm glass tubes, and sequenced through 5 cycles as described by Kuhn and Crabb (1986). The final product was dried under $N_2$ and stored at 4 °C prior to HPLC detection.

Phenylthiohydantoin (PTH) derivatives were separated by reverse phase HPLC as described by Bhown and Bennett (1985). Elution was monitored at 254 nm, and PTH-Glu peaks were collected, dried under a stream of filtered air, and suspended in 0.7 mL of TS-1 followed by 3 mL of Bio-Safe II. Linear regression analysis was carried out on the peak height of a standard curve, and nanomoles and amount of PTH-Glu were calculated.

*Mammalian Cell Culture*. The bovine hepatic cell line LB9Li was obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The rat osteosarcoma 17/2 (ROS 17/2) cell line was grown in Coon's F-12 medium containing 10% fetal bovine serum and 0.6 μg/mL amphotericin B. Both lines were grown at 37 °C in an atmosphere of 95% air and 5% $CO_2$. Following passage, cells were refed on the second day and harvested on the fourth or fifth day upon reaching confluency. A total of 12−16 confluent 100 mm plates were rinsed with ice-cold SI buffer (0.25 M sucrose/0.025 M imidazole/pH 7.3) and mechanically harvested in the same buffer. The cell suspension was pelleted at low speed, and the pellet was solubilized by suspension in 1 mg/mL phosphatidylcholine/1 mg/mL CHAPS/4 mM DTT and dispersion with three strokes in a glass on glass dounce homogenizer. Solubilization proceeded for 30 min on ice with occasional mixing. Protein was quantitated by the Lowry method (1951), and aliquots were immediately frozen in liquid nitrogen.

*Reagents*. Tissue solubilizer, Bio-Safe II, and Fluoro-Hance were obtained from Research Products International (Mt. Prospect, IL). HPLC grade pyridine (PYR), phenyl isothiocyanate (PITC), triethylamine (TEA), trifluoroacetic acid (TFA), and 6 N constant-boiling HCl were obtained from Pierce (Rockford, IL). Burdick and Jackson HPLC grade acetonitrile and methanol were obtained from Baxter (McGaw Park, IL). Phosphatidylcholine, polyvinylpyrrolidone, TPCK-treated trypsin, pharmalytes (pH 4.0−6.5 and pH 2.5−5.0), 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), Coon's F-12 medium, Dulbecco's modified Eagle's medium (DME), amphotericin B, penicillin G, streptomycin sulfate, and CNBr-preactivated Sepharose 4B were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum was obtained from HyClone Laboratories (Logan, UT). TC100 medium and lipofectin were obtained from Gibco BRL/Life Technologies (Grand Island, NY). Tryptose broth was obtained from DIFCO (Detroit, MI). Sodium [$^{14}$C]bicarbonate was obtained from ICN Biochemicals (Costa Mesa, CA). Phylloquinone was obtained from Sigma and subsequently reduced to the hydroquinone according to the procedure of Wood and Suttie (1988).

## RESULTS

*Expression and Processing of Human and Bovine preproBGP*. Human and bovine preproBGP cDNAs were each cloned into the transfer vector pEVbsmer, and recombinant baculoviruses were obtained by cotransfecting the transfer vectors with wild-type baculovirus (AcNPV DNA) into Sf21 cells. These recombinant baculoviruses were plaque-purified and used for recombinant protein expression. Protein expression was determined by immunoblot analysis of whole cell extracts and the media. Three distinct bands were seen in cells infected with each of the recombinant viruses: a minor faint higher doublet of bands and a lower, more predominant band (Figure 1A). The doublet of higher bands, which was very faint in the case of bovine BGP expression, was assumed to be preproBGP ($MW_r$ = 12 kDa). The presence of two bands may be due to partial proteolysis. The major lower band ran above mature BGP and heat-decarboxylated BGP (dBGP) ($MW_r$ = 5.8 kDa) (Figure 1), indicating negligible processing of the propeptide, and hence the band was assumed to be proBGP ($MW_r$ = 9 kDa). The Sf21 cell media also contained this 9 kDa protein with no sign of the mature protein (Figure 1B). Whether the extracellular proBGP was the result of cell lysis and/or cell secretion was not determined with any certainty.

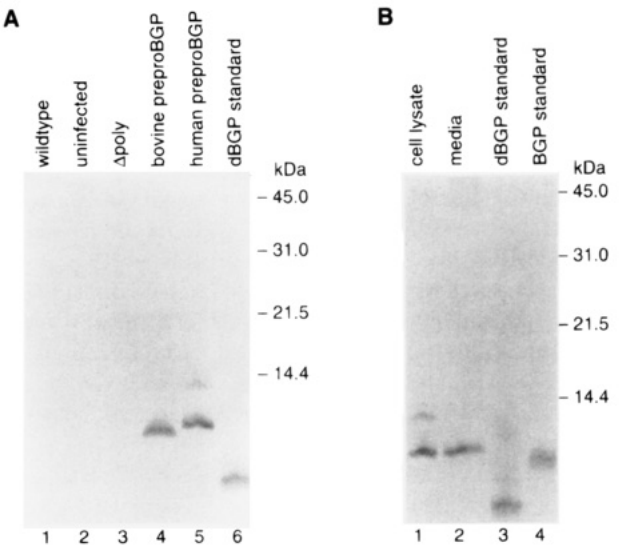


FIGURE 1: (A) Immunoblot of bovine and human preproBGP produced and processed in Sf21 cells. Whole cell lysates harvested 48 hpi were subjected to 17.5% SDS−PAGE and subsequently immunoblotted. Shown are cells infected with wild-type baculovirus (lane 1), uninfected Sf21 cells (lane 2), polyhedrin-deleted baculovirus (lane 3), recombinant bovine preproBGP baculovirus (lane 4), recombinant human preproBGP baculovirus (lane 5), and purified heat-decarboxylated BGP (dBGP) as a marker (lane 6). (B) Immunoblot of media from recombinant bovine preproBGP baculovirus-infected cells (lane 2). Cell lysate prepared from the recombinant bovine-infected cells is shown for comparison (lane 1). Purified bovine BGP and heat-decarboxylated BGP (dBGP) are indicated (lanes 3 and 4).

Although both the human and bovine proteins were produced in roughly the same amounts (based on immunoblot), the human protein was not processed to the same extent as the bovine protein. As estimated from the immunoblot, 75% of the human preproBGP was converted to proBGP, whereas greater than 95% of the bovine protein was processed to proBGP. These same bands were not seen in extracts from uninfected cells or in cells infected with either wild-type baculovirus or polyhedrin-deleted baculovirus (Figure 1A). Accumulation of the expressed proteins peaked at 48 hpi and remained stable to at least 72 hpi (data not shown). More complete processing of the human preproBGP

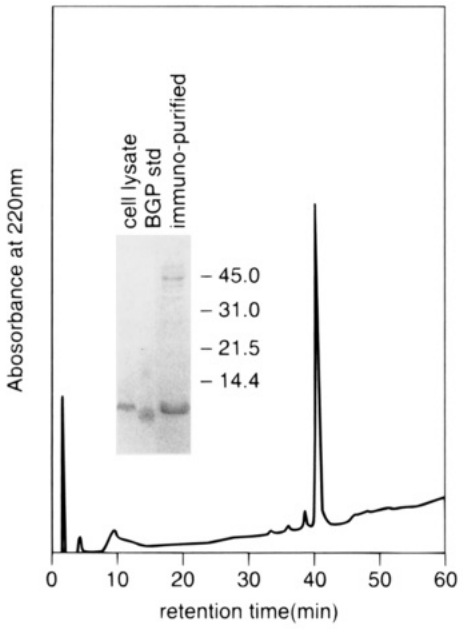


FIGURE 2: HPLC profile of immunoaffinity-purified bovine proBGP. Mobile phase: buffer A, 0.1% trifluoroacetic acid (TFA) in (2:98 v/v) acetonitrile/$H_2O$; buffer B, 0.1% TFA in (60:40, v/v) acetonitrile/$H_2O$. Flow rate, 1.0 mL/min; temperature, 25 °C. Following injection of the sample onto a Hamilton PRP-1 analytical column, a linear gradient from 0 to 100% B was developed over 1 h. The insert shows a blot of bovine proBGP immunoaffinity-purified from cell lysate for N-terminal sequence analysis. Recombinant bovine preproBGP baculovirus-infected cells were harvested 48 hpi, and cell lysates were prepared. ProBGP was purified from the lysate with an anti-BGP antibody resin, subjected to 17.5% SDS−PAGE, transferred to an Immobilon-P membrane, and Coomassie stained. Purified bovine BGP and cell lysate containing the recombinant bovine proBGP were used as markers in the same gel, except they were visualized by immunoblot.

to proBGP was not seen at the later time point. The differential processing efficiencies of the human and bovine leader sequences may be explained by the low sequence homology between the two peptides, especially near the leader cleavage site (Kiefer *et al.*, 1990). Because the bovine recombinant was more completely processed to the pro form, it was utilized in further experimentation. Attempts to increase the yield of proBGP by expression in the cellular cytosol with a construct lacking a leader sequence were unsuccessful. The expressed protein levels peaked at 36 hpi but were unstable and greatly decreased at 72 hpi (data not shown).

*Isolation and Characterization of Bovine proBGP.* Quantitation of bovine proBGP was carried out by radioimmunoassay (RIA) of detergent-solubilized cell lysate and cell media. Approximately 40−50 $\mu$g of proBGP/mL of cell lysate [$1 \times 10^7$ cells $mL^{-1}$ (100 mm plate)$^{-1}$] was found on a routine basis at 48 hpi; the amount of proBGP present in the media at this time (per 100 mm plate) was only ~50% of this amount, and the recombinant protein was selectively isolated from the cell lysates. Purification of proBGP from the cell lysate was performed using anti-BGP antibody resin; a 50% recovery was routinely obtained (data not shown). When the immunoaffinity-purified proBGP was analyzed by reversed phase HPLC, a single peak was seen (Figure 2). To confirm the identity of the expressed bovine BGP precursor as proBGP, N-terminal sequencing of the antibody-purified protein was carried out. The sequence of the first

Table 1: *In Vitro* Carboxylation of Crude Recombinant Bovine proBGP[a]

| substrate source | carboxylase act. (net dpm) |
|---|---|
| recombinant cell lysate | 59295 |
| wild-type cell lysate | 565 |
| recombinant cell lysate minus microsomes | 19 |
| recombinant cell media | 7245 |

10 amino acids was determined from an Immobilon-bound Coomassie-stained band (Figure 2). Sequences of two proteins were found, a major sequence (X-P-G-D-A-E-S-G-K-G-) and a minor sequence (G-D-A-E-S-G-K-G-A-X-), which differed only by their two most N-terminal residues. The sequence of the major protein coincided with the published sequence (K-P-G-D-A-E-S-G-K-G-A-A-) of the bovine BGP propeptide (Kiefer *et al.*, 1990). The minor protein was similar, except its sequence started two residues from the N-terminus of the published sequence. Differential processing of the leader peptide or partial proteolysis of the propeptide may account for the disparity.

The ability of the expressed proBGP to serve as a substrate for the vitamin K-dependent carboxylase was analyzed by *in vitro* carboxylation assay. An unfractionated cell lysate prepared from a single large-scale infection ($8 \times 10^7$ cells) was incubated with detergent-solubilized bovine liver microsomes. The recombinant bovine preproBGP baculovirus-infected cell lysate and the conditioned media were both found to contain a carboxylatable substrate as monitored by the incorporation of $^{14}CO_2$ in the presence of solubilized bovine liver microsomes (Table 1). No carboxylatable substrate was detected in cell lysates of wild-type baculovirus-infected cells. Although considered unlikely, it is possible that the Sf21 cells contained the carboxylase enzyme and that the protein assumed to be rdproBGP was in fact largely carboxylated. BGP and dBGP exhibit different mobilities on SDS−PAGE, and any change in the carboxylation status of dproBGP should also be detectable by a band shift. Recombinant bovine preproBGP baculovirus-infected cells in both serum-containing and serum-free media were grown with and without vitamin K supplementation (10 $\mu$g/mL media). Following SDS−PAGE with subsequent immunoblot analysis of whole cell extracts, the mobility of proBGP was found to be unaltered in the presence of vitamin (Figure 3), confirming the recombinant protein as bovine des-$\gamma$-carboxylated proBGP (rbdproBGP).

*Substrate Activity of dBGP and rbdproBGP.* Heat-decarboxylated bovine BGP (dBGP) and immunopurified rbdproBGP were compared as substrates for the vitamin K-dependent carboxylase. The proteins were each quantitated by RIA using the same standard curve, and an equimolar amount of each substrate (0.67 $\mu$M) was incubated with detergent-solubilized bovine liver microsomes. Carboxylation of rbdproBGP was 2 times higher than that of dBGP after both 10 and 60 min of incubation (data not shown). The $K_{m(app)}$ for rbdproBGP in a semipurified bovine carboxylase preparation was found (Figure 4) to be 237 nM, and in the same system, the $K_{m(app)}$ for dBGP was 720 nM.

*Analysis of Carboxylated dBGP: Isoelectric Focusing.* Analysis of the multi-site-specificity of the carboxylase was first approached by characterization of the carboxylated dBGP isolated from a reaction mixture. Using dBGP and BGP as markers for the fully uncarboxylated substrate and fully carboxylated species, respectively, two distinct bands

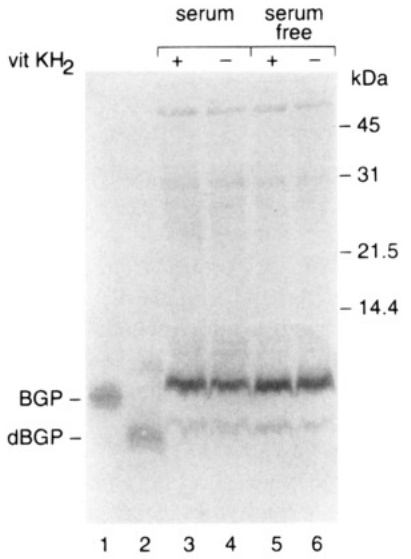


FIGURE 3: Effect of reduced vitamin K supplementation on the carboxylation status of baculovirus-expressed rbproBGP. Infected cells in serum-containing or serum-free media, with or without added vitamin $KH_2$ (10 $\mu$g/mL media), were harvested at 48 hpi, and cell extracts were subjected to 17.5% SDS−PAGE followed by immunoblot analysis. Purified bovine BGP and heat-decarboxylated BGP (dBGP) standards are indicated (lanes 1 and 2).

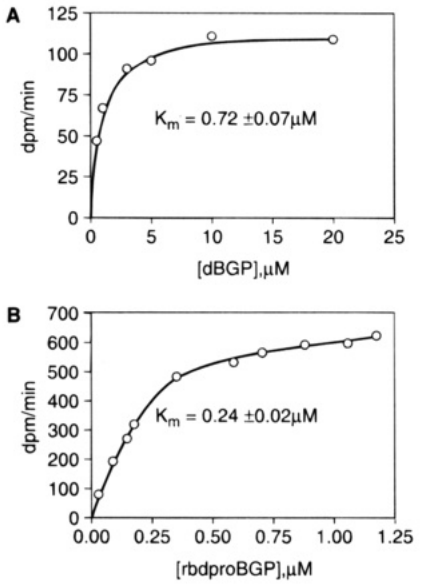


FIGURE 4: Initial rate of carboxylation as a function of dBGP (A) and rbdproBGP (B) concentration. Incorporation of $^{14}CO_2$ in the presence of 500-fold-purified vitamin K-dependent carboxylase containing 100 $\mu$M hFX propeptide was quantitated following a 10 min incubation with varying concentrations of rbdproBGP as described under Materials and Methods (carboxylation was predetermined to be linear for at least 10 min). Each value represents the mean of duplicate measurements. The $K_m$ values shown were calculated by fitting initial rate data to the hyperbola $V = VA/(K + A)$ using the Cleland (1977) program HYPER.

representing mono- and dicarboxylated BGP were seen following a 60 min incubation of 20 $\mu$M dBGP with the semipurified enzyme. The same relative distribution of these two species was seen as early as 30 min of incubation and also at 180 min (Figure 5). The distributions of the partially carboxylated BGP products were also quantitated with a Betagen Blot Analyzer, and at each time point, the mono- and di-Gla bands were found to contain nearly equivalent radioactivity, indicating a 2:1 ratio of mono- to dicarboxylated BGP. Fully carboxylated BGP did not accumulate to a significant level, and even after a 15 h incubation, the same ratio of mono- and di-Gla species was seen, with a negligible amount of the fully carboxylated species (data not shown). Carboxylation of 5 $\mu$M dBGP with solubilized bovine liver microsomes revealed a similar pattern of carboxylation (Figure 6), indicating no significant difference in the specificity of carboxylation between the crude and semipurified enzyme preparations. However, at lower, nonsaturating (1 and 0.5 $\mu$M) concentrations of the dBGP substrate, only the di-Gla product was seen following a 60 min incubation (Figure 6).

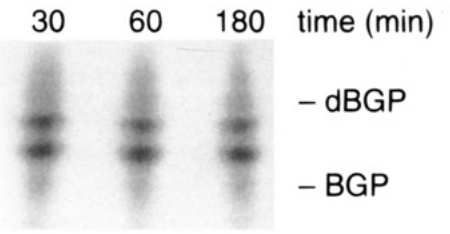


FIGURE 5: Isoelectric focusing of carboxylated dBGP. dBGP (20 $\mu$M) was carboxylated for 30, 60, and 180 min with 500-fold-purified bovine liver carboxylase. The carboxylated species were isolated as described under Materials and Methods, resolved on an IEF gel (pH 2.5−5.0), and subjected to fluorography. Positions of BGP and dBGP markers, detected by Coomassie staining of the same gel, are indicated. $^{14}CO_2$ incorporation into the mono- and di-Gla species was quantitated with a Betagen blot analyzer. The ratio of $^{14}$C-mono-Gla to $^{14}$C-di-Gla was 49:51 at 30 min, 47:53 at 60, and 44:56 at 180 min, indicating a 2:1 ratio of mono- to dicarboxylated product.

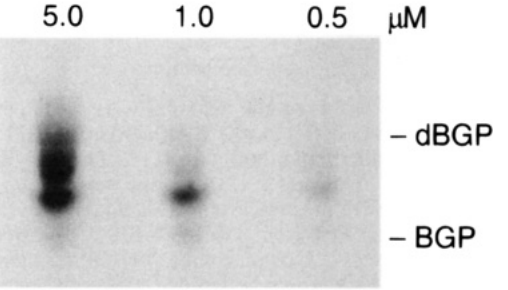


FIGURE 6: Effect of dBGP concentration on the products of dBGP carboxylation. Assays containing 0.5, 1, and 5 $\mu$M dBGP were carried out for 60 min with solubilized bovine liver microsomes and 50 $\mu$M free propeptide, and the carboxylated species were isolated as described in Figure 5. Half of the reaction products from the 5 $\mu$M incubation and all of the reaction products from the 0.5 and 1 $\mu$M incubations were loaded onto the gel.

*Analysis of Carboxylated rdproBGP: Isoelectric Focusing.* The natural substrate for the carboxylase contains a covalently bound propeptide, and the influence of this alteration on the products formed *in vitro* was studied by utilizing the recombinant dproBGP. This substrate was carboxylated by solubilized bovine liver microsomes in an assay supplemented with 50 $\mu$M free hFX propeptide, and the carboxylated reaction products were isolated. Following a 90 min incubation, a single predominant band was seen in the fluorogram of IEF-separated products along with a faint band of slightly lower p*I* (Figure 7). Identification of the Gla content of these products was determined by partial heat decarboxylation and electrophoresis of an identical pool of $^{14}$C-rproBGP reaction products. Decarboxylation of $^{14}$C-labeled Gla will leave $^{14}$C-labeled Glu residues with half the specific radioactivity, and a ladder of three bands was

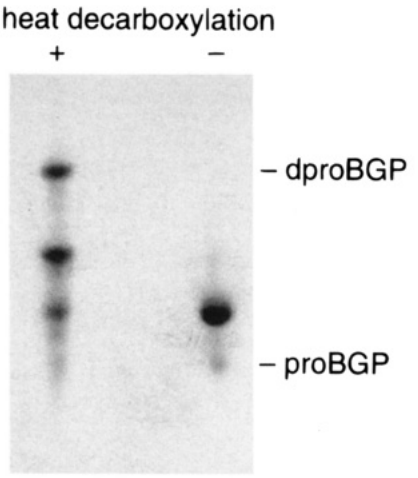


FIGURE 7: Isoelectric focusing of carboxylated rdproBGP. Carboxylation of 0.8 $\mu$M rdproBGP was carried out for 90 min with solubilized bovine liver microsomes and 50 $\mu$M free propeptide. Carboxylated reaction products were isolated as described under Materials and Methods, resolved on an IEF (pH 4.0−6.5) gel, and subjected to fluorography. A similar pool of carboxylated products was partially heat-decarboxylated to provide a ladder of partially carboxylated $^{14}$C-rproBGP species.

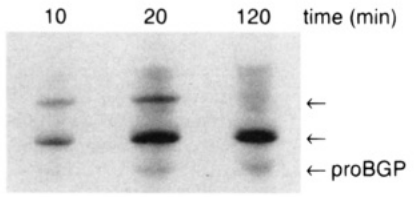


FIGURE 8: Time course of carboxylation of rbdproBGP. Reactions were carried out in solubilized bovine liver microsomes containing 50 $\mu$M hFX propeptide and 0.8 $\mu$M rdproBGP. The carboxylated products were isolated as described in Figure 7. All of the isolated material from the 10 and 20 min reactions but only half of the isolated material from the 120 min reaction was loaded onto the gel.

observed. The three bands were therefore identified, in order of decreasing p*I*, as fully uncarboxylated rdproBGP (top band), monocarboxylated rproBGP (middle band), and dicarboxylated rproBGP (bottom band). On the basis of these markers, the predominant $^{14}$C-rproBGP carboxylation product seen in Figure 7 was identified as dicarboxylated rproBGP and the faint band just below it as fully carboxylated rproBGP. This same distribution of partially carboxylated species was seen in numerous carboxylase assays of rdproBGP. In order to determine if some rproBGP was processed to dBGP during incubation with solubilized crude liver microsomes, BGP and dBGP were electrophoresed along with $^{14}$C-rproBGP. Due to the basicity of the propeptide, the p*I* of proBGP is much higher than that of BGP, and a clear distinction can be made between the two proteins on an IEF gel. Processing of rproBGP to BGP was never observed (data not shown).

Assay of rdproBGP incubations revealed that carboxylation was complete after 1 h of incubation (data not shown). The distribution of carboxylated rproBGP species was therefore monitored at earlier time points. Following a 10 min incubation, the majority of the products were dicarboxylated; however, a small amount of monocarboxylated product was observed. This same distribution was seen at 20 min, but by 2 h all of the mono-Gla product had been converted to di-Gla product (Figure 8). The tri-Gla product appeared to

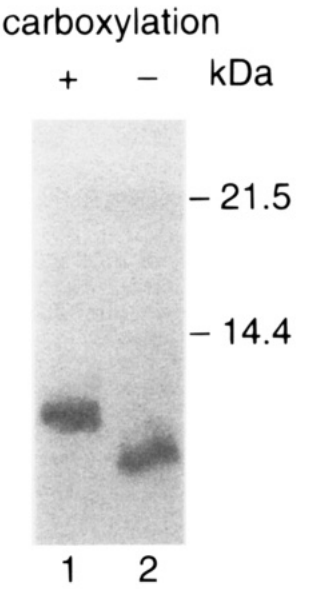


FIGURE 9: Immunoblot of carboxylated rdproBGP. Carboxylation of 0.7 $\mu$M immunoaffinity-purified rdproBGP was carried out with solubilized bovine liver microsomes in the presence of 50 $\mu$M free propeptide for 2 h. Products were isolated as described under Materials and Methods and subjected to 17.5% SDS−PAGE followed by immunoblot analysis. Shown are carboxylated (lane 1) and uncarboxylated (lane 2) rdproBGP.

increase with time, although remained <10% of the total species. Following an extended 15 h incubation (data not shown), the amount of tricarboxylated species was increased over that seen after 2 h but did not exceed 20% of the total carboxylated species. The same distribution of products was obtained using the semipurified enzyme preparation rather than solubilized microsomes.

The extent of carboxylation of rdproBGP substrate was also determined by immunoblot analysis. After a 2 h incubation, carboxylated products in the incubation were isolated and subjected to SDS−PAGE. Following an immunoblot of the gel, the carboxylated rdproBGP reaction products were found to migrate in a single band of lesser mobility than uncarboxylated rdproBGP (Figure 9). These data show that the entire pool of recombinant protein was carboxylated under the reaction conditions used, and that all of the rdproBGP was converted to the predominant dicarboxylated product within 2 h.

The possible influence of tissue differences in the specificity of the carboxylase was also investigated. Carboxylation of rdproBGP was investigated in solubilized extracts of a rat osteosarcoma cell line (ROS 17/2) known to contain a functional vitamin K-dependent carboxylase (Nishimoto & Price, 1985) and a bovine liver (LB9Li) cell line. Utilizing the solubilization and incubation procedures described under Materials and Methods, the vitamin K-dependent carboxylation of dproBGP in 3 h in the ROS 17/2 cell extract was $13.1 \times 10^3$ dpm and in LB9Li cells was $11.6 \times 10^3$ dpm. Reaction products isolated after 3 h of carboxylation with each cell preparation were found to consist of both mono- and di-Gla species which were roughly equivalent in size, indicating a 2:1 ratio of mono- to dicarboxylated rproBGP (Figure 10). These data indicate that the bone and liver mammalian cell culture carboxylase activities carboxylate rdproBGP to the same extent.

*Carboxylation of dBGP and rdproBGP: Gla Site Determination.* The location of Gla residues in partially carboxy-

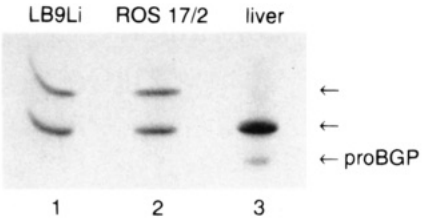


FIGURE 10: Products of rdproBGP carboxylation by extracts of cultured liver (LB9Li) or bone (ROS 17/2) cells. Carboxylase assays were carried out as described under Materials and Methods for 3 h. Reaction products were isolated, resolved on an IEF gel, and subjected to fluorography as described in Figure 7. Products of carboxylation by solubilized liver microsomes are shown for comparison. The arrow indicates in descending order mono-, di-, and tricarboxylated rproBGP.

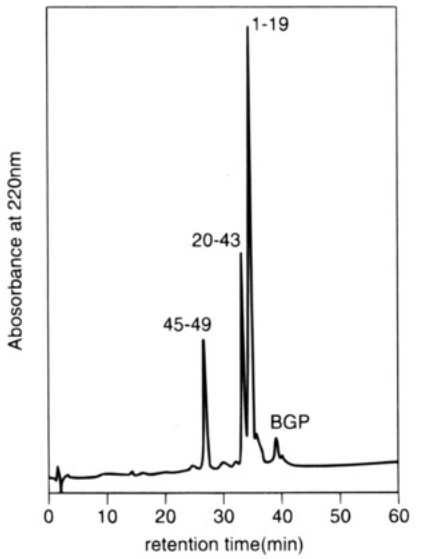


FIGURE 11: Reverse phase HPLC separation of trypsin-digested BGP. Samples were resolved under conditions described under Materials and Methods. Identification of the peaks was determined by partial manual Edman degradation of fragment 20−43. Peaks containing fragments 1−19 and 20−43 were manually collected for analysis of Gla content.

lated rdproBGP and dBGP reaction products was determined by tryptic cleavage of the products. The resulting tryptic fragments, containing residues 1−19, 20−43, and 45−49, were separated by reverse phase HPLC (Figure 11). Fragment 1−19, which contains Glu site 17, and fragment 20−43, which contains Glu sites 21 and 24, were collected and acid-hydrolyzed, during which all Gla residues were converted to Glu residues, and the amino acids were subjected to phenylthiocarbamyl (PTC-Glu) analysis. The specific activities of Glu site 17 and the combined Glu sites 21 and 24 were determined and found to be highest for PTC-Glu 21+24, with both dBGP and rdproBGP substrates (Table 2). In the case of rdproBGP, >90% of the liver microsomal carboxylated products were dicarboxylated (Figure 8), indicating preferential carboxylation at sites 21 and 24. The same Glu site-specificity of the carboxylase (~10% site 17, and 90% sites 21 and 24) was also observed (data not shown) for the products of the carboxylase activity found in cultured bone and liver cells shown in Figure 10, suggesting no difference in site-specificities between carboxylase activities from bone and liver.

The data in Figure 8 indicate that the propeptide-containing substrate (rdproBGP) was rapidly converted to a predominant

Table 2: Analysis of Gla in $^{14}$C-BGP Tryptic Fragments[a]

| substrate | concn ($\mu$M) | incubation time (h) | % $^{14}$C-PTC-Glu | |
|---|---|---|---|---|
| | | | $Gla_{17}$ | $Gla_{21+24}$ |
| dBGP | 5 | 1 | 11 | 89 |
| | 20 | 1 | 13 | 87 |
| dproBGP | 0.7 | 2 | 13 | 87 |
| | 0.7 | 15 | 12 | 88 |

[a] Carboxylated dBGP and dproBGP were analyzed as described under Materials and Methods. Tryptic fragments 20−43 and 1−19 were acid hydrolyzed and subjected to PTC-Glu analysis. The specific activities of $Glu_{17}$ and $Glu_{21+24}$ were determined and expressed as a percentage of the combined specific activities. The calculated specific activity of $Glu_{21+24}$ was multiplied by a factor of 2 to calculate total incorporation of $^{14}CO_2$ rather than the average specific activity of both sites.

Table 3: Distribution of $^{14}$C-Gla in Carboxylated dBGP[a]

| expt | Glu site | sp act. of residue (dpm/nmol) | % of total |
|---|---|---|---|
| 1 | 17 | 98 | 13 |
| | 21 | 255 | 33 |
| | 24 | 417 | 54 |
| 2 | 17 | 120 | 16 |
| | 21 | 225 | 29 |
| | 24 | 422 | 55 |

[a] Two separate reactions were incubated for 60 min each. The specific activity (dpm/nmol) was determined at site 17 by analysis of tryptic fragment 1−19. Specific activities of sites 21 and 24 were determined by manual Edman degradation of tryptic fragment 20−43 as described under Materials and Methods. The incorporation of $^{14}CO_2$ at each Gla site is also expressed as a percentage of the total combined specific activities per experiment.

di-Gla product, and Table 2 indicates that this di-Gla product was mainly 21,24-di-Gla product. In the case of the dBGP products where two-thirds of the products were mono-Gla, the specificity could not be determined by simple analysis of the tryptic peptides. Tryptic fragment 20−43, which contains Glu sites 21 and 24 from a dBGP incubation, was therefore sequenced five cycles using manual Edman degradation to determine the specific activity at each site. In two separate experiments, analyzing the mono- and di-Gla species combined, it was found that site 24 was most highly carboxylated, followed by site 21 and then site 17 (Table 3).

*Effect of Free Propeptide on Carboxylation of rdproBGP and dBGP*. Data on the multi-site-specificity of the carboxylation of rdproBGP and dBGP were obtained in the presence of free propeptide, which stimulates the rates of carboxylation of both substrates. Carboxylation was also carried out in the absence of free propeptide to determine the effect, if any, of free propeptide on the pattern of carboxylation of each substrate. Free propeptide was found to have no effect on the distribution of carboxylated species of dBGP; approximately equal amounts of mono- and dicarboxylated BGP accumulated in a 60 min incubation (Figure 12A). This same distribution of species was seen with the partially purified enzyme, both with and without additional propeptide supplementation. In the case of rdproBGP (Figure 12B), mono- and di-Gla products were seen in approximately a 1:1 ratio along with a trace amount of tri-Gla product following a 2 h incubation in the absence of free propeptide. Following a 15 h incubation, under the same reaction conditions, the di-Gla product was the predominant species; however, a significant amount of mono-

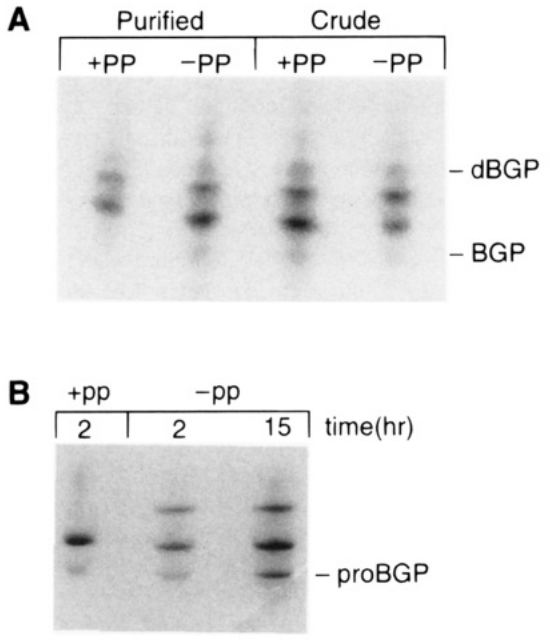


FIGURE 12: Effect of free propeptide on carboxylation of dBGP and rdproBGP. (A) Carboxylation of 5 $\mu$M dBGP was carried out with either partially purified bovine- or detergent-solubilized (crude) bovine liver microsomes in the presence and absence of 100 $\mu$M propeptide. Following 60 min incubations, the carboxylated products were isolated, separated on an IEF gel, and subjected to fluorography as described in Figure 5. The mobilities of dBGP and BGP markers, as indicated by Coomassie staining of the same gel, are indicated. Quantitation utilizing a Betagen blot analyzer indicated that under all conditions the mono-Gla product represented 53−54% of the total. (B) Carboxylation of 0.8 $\mu$M rdproBGP was carried out with solubilized bovine liver microsomes in the absence of free propeptide for 2 and 15 h or in the presence of 50 $\mu$M hFX propeptide for 2 h. Carboxylated products were isolated; aliquots were resolved on an IEF gel and subjected to fluorography as described in Figure 7.

and tri-Gla products was also present. In contrast, following a 2 h incubation in the presence of free propeptide, only di-Gla product was seen along with a trace amount of tri-Gla product. These data suggest that free propeptide alters the pattern of carboxylation of rdproBGP. In the presence of free propeptide, the mono-Gla product has a higher affinity for the enzyme and is readily converted to di-Gla product, whereas in the absence of free propeptide, mono-Gla product accumulated with time, and a significant amount was still present after 15 h.

## DISCUSSION

These studies have demonstrated that a physiological substrate for the vitamin K-dependent $\gamma$-glutamyl carboxylase can be produced in insect cells utilizing a baculovirus expression system. Expression of preproBGP resulted in proBGP as a major product which was an excellent substrate for the carboxylase enzyme. Small peptides containing Glu-Glu sequences are normally used to assay this enzyme, and they typically exhibit $K_m$ values of a few millimolar (Rich *et al.*, 1981). Addition of a covalently bound propeptide to these peptides (Ulrich *et al.*, 1988; Hubbard *et al.*, 1989) lowers the $K_m$ to a few micromolar, and a 59 residue peptide containing the propeptide and entire uncarboxylated Gla region of human factor IX (Wu *et al.*, 1990) has been reported to have a $K_m$ of 0.55 $\mu$M. The proBGP described in this report represents a normal substrate of the enzyme and has a $K_m$ of 0.24 $\mu$M. Although these apparent $K_m$'s are undoubtedly influenced by incubation conditions and the presence or absence of free propeptide (Knobloch & Suttie, 1987; Cheung *et al.*, 1990), it is apparent that one role of the propeptide domain of these proteins is to increase the affinity of the enzyme for its substrate. The studies reported here were directed toward determining whether or not this domain also influenced the specificity and directionality of the carboxylation reaction.

Analysis of small peptides with Glu-Glu sequences (Finnan & Suttie, 1980; Decottignies-Le Marechal *et al.*, 1979) has demonstrated that the most amino-terminal Glu is preferentially carboxylated, and analysis of partially $\gamma$-carboxylated pools of bovine prothrombin produced *in vivo* (Liska & Suttie, 1988) has also indicated a preference for carboxylation at the most amino-terminal Glu sites. In these studies, carboxylation of high concentrations (5 or 20 $\mu$M) of dBGP resulted in the formation of mono- and di-Gla products at a 2:1 ratio. This ratio did not change with time, and there was no evidence of a lag in di-Gla BGP formation which required a pool of mono-Gla product for its formation. Rather, the data suggest that in the presence of saturating substrate, about 65% of the initial carboxylation events result in mono-Gla formation while 35% of the time a second carboxylation event occurs, followed by di-Gla release. Essentially no tri-Gla product was formed. Carboxylation occurred approximately 15% at residue 17, 30% at residue 21, and 55% at residue 24. Carboxylation of nonsaturating concentrations of dBGP resulted in an increased formation of di-Gla product, suggesting that the mono-Gla product competes effectively with dBGP for further carboxylation and at low dBGP concentrations mono- to di-Gla conversion occurs.

The presence of the covalently bound propeptide has been shown to be essential for normal *in vivo* carboxylation of the vitamin K-dependent proteins (Katow *et al.*, 1993), and it also influenced *in vitro* carboxylation. Carboxylation of dproBGP yielded, as did dBGP, a predominantly di-Gla product, but in this case significant tri-Gla product was formed with time, and mono-Gla product formed early in an incubation was subsequently subjected to further carboxylation. The sequence-specificity of the reaction was, however, not altered by the propeptide; the extent of conversion of Glu to Gla sites was again Glu 24 > Glu 21 > Glu 17.

The carboxylation data presented were obtained in the presence of free propeptide, which has previously been shown to increase the rates of substrate carboxylation by lowering the apparent $K_m$ of the Glu site substrate (Knobloch & Suttie, 1987). Under conditions where the enzyme was saturated with dBGP, free propeptide was found to have no effect on the distribution of carboxylated species. However, under conditions where rdproBGP was less than saturating, free propeptide produced a marked alteration. In the presence of free propeptide, only di- and tri-Gla rproBGP were seen, whereas in its absence mono-, di-, and tri-Gla rbproBGP accumulated. These data suggest that free propeptide increases the affinity of the carboxylase for the propeptide-linked mono-Gla product and prevents it from disassociating prior to an additional carboxylation event. The physiological significance of this response is not clear.

Although most BGP species are thought to be fully carboxylated *in vivo* (Hauschka *et al.*, 1989), undercarboxy-

lation of site 17 in BGP has been reported. Poser *et al.* (1980) found that human BGP isolated from the bone of a 76-year-old male was fully carboxylated at sites 21 and 24 but only 9% carboxylated at site 17, and Cairns and Price (1994) have now shown that significant undercarboxylation of Glu 17 is present in human adults, in neonates, and in fetal bone. Site 17 has also been reported to be uncarboxylated in wallaby BGP (Huq *et al.*, 1984), and bluegill BGP was found to contain an uncarboxylated Glu residue amino-terminal to a fully carboxylated Gla 17 (Nishimoto *et al.*, 1992). This is the first example of such an occurrence in a BGP species, and suggests that multiple factors are involved in targeting Glu sites as substrates for $\gamma$-carboxylation.

The predominant carboxy-terminal Glu site-specificity of the enzyme when carboxylating dBGP and dproBGP is in contrast to the carboxylation of small peptide substrates and *in vivo* prothrombin carboxylation. This may reflect a unique order of carboxylation for dBGP relative to the vitamin K-dependent clotting factors. This might not be surprising since the clotting factors and BGP share little sequence homology. The clotting factors are much larger and have multiple (9−12) paired Gla residues compared to BGP, which is about one-tenth the molecular size and has three Gla sites separated by more than one residue. However, two of the three Glu substrate sites in BGP are within the EXXXEXC sequence which is the one invariant sequence common to all known Gla-containing proteins (Price *et al.*, 1987). This, along with the propeptide region, may be very important in the substrate/enzyme interaction, and the possible involvement of the cysteine in this consensus sequence in substrate recognition has also been considered (Price, 1988). The two most extensively carboxylated residues, 21 and 24, flank this residue, and the extent of their carboxylation may be influenced by it.

How multiple Glu residues are processed *in vivo* is unknown. Certainly, continuous carboxylation of multiple potential Gla sites, without dissociation of the partially carboxylated protein, would be the most efficient method by which a multiple processing event could proceed. Compared to this "ideal" model of *in* vivo carboxylation, the *in vitro* carboxylation studied here was found to be highly inefficient. With dBGP as a substrate, a lag in the formation of the di-Gla product did not occur, suggesting that dicarboxylation was occurring without dissociation of the mono-Gla species. However, sequential carboxylation of dBGP to yield the di-Gla product represented only about one-third of the total carboxylation events. This response was strongly influenced by the presence of the propeptide. Using rd-proBGP as a substrate, a small amount of mono-Gla product was seen to accumulate within the first 20 min of the reaction but was completely converted to di-Gla product within 90 min. Since the majority of the products were di-Gla species in as little as 10 min, it is likely that dicarboxylation is a predominantly continuous event with mono-Gla product occasionally dissociating from the enzyme. Whether or not the bound propeptide directs tighter enzyme/substrate interaction, leading to fewer incidents of mono-Gla product dissociating from the enzyme, is unknown; such a role for the propeptide seems plausible given the lower $K_m$ obtained for rdproBGP versus dBGP.

One possible explanation for the failure of the $\gamma$-carboxylase to fully carboxylate all three target glutamate residues during the lifetime of the BGP−carboxylase complex could be that the reaction conditions used for the *in vitro* carboxylation reaction are not optimal. The carboxylase assay has been previously optimized to give the highest rate of $\gamma$-carboxyglutamate formation per carboxylase molecule. This optimization strategy favors conditions under which the lifetime of the carboxylase substrate complex is short so that each enzyme molecule can partially carboxylate a large number of substrate molecules. In contrast, complete carboxylation of each BGP molecule bound to the carboxylase requires that the lifetime of the complex be far longer, and the absolute rate of $\gamma$-carboxyglutamate formation per carboxylase will therefore be much lower. These considerations suggest that the *in vitro* conditions needed to reproduce the *in vivo* ability of the carboxylase to fully carboxylate the target glutamate residues in a substrate protein will only be found when the carboxylase is assayed for its ability to fully carboxylate each substrate protein rather than for its ability to make the maximal overall amount of $\gamma$-carboxyglutamate. The isoelectric focusing gel procedure developed here provides a direct assay which can be used to identify the conditions that are needed to achieve complete carboxylation without the generation of partially carboxylated substrate proteins.

It is possible that an *in vivo*, a multiprocessing event might require a helper protein that prevents premature dissociation of the enzyme/substrate complex and hence promotes processive carboxylation. An involved chaperone might also function by modulating protein folding, thereby rendering specific Glu residues more accessible to the catalytic site. The similarity of product formation seen with either crude microsomes or a 500-fold purified preparation of the enzyme would, however, argue against the requirement for a second protein. These data do, however, provide information on the directionality of carboxylation of at least one substrate for this unique enzyme, and have provided an approach to further experimentation.

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## REFERENCES

Bergman, T., Carlquist, M., & Jornvall, H. (1986) in *Advanced Methods in Protein Microsequence Analysis* (Wittmann-Liebold, B., Ed.) pp 45−46, Springer-Verlag, Berlin, Heidelberg.

Berkner, K. L., Harbeck, M., Lingenfelter, S., Bailey, C., Sanders-Hinck, C. M., & Suttie, J. W. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6242−6246.

Bhown, A. S., & Bennett, J. C. (1985) *Anal. Biochem. 150*, 457−462.

Borowski, M., Furie, B. C., & Furie, B. (1986) *J. Biol. Chem. 261*, 1624−1628.

Cairns, J. R., & Price, P. A. (1995) *J. Bone Miner. Res.* (in press).

Cheung, A., Suttie, J. W., & Bernatowicz, M. (1990) *Biochim. Biophys. Acta 1039*, 90−93.

Cleland, W. W. (1977) *Adv. Enzymol. Relat. Exp. Mol. Biol. 45*, 273−387.

de Boer-van den Berg, M. A. G., Vitendaal, M. P., & Vermeer, C. (1987) *Mol. Cell. Biochem. 75*, 71−76.

Decottignies-Le Marechal, P., Rikong-Adie, H., & Azerad, R. (1979) *Biochem. Biophys. Res. Commun. 90*, 700−707.

Finnan, J. L., & Suttie, J. W. (1980) in *Vitamin K Metabolism and Vitamin K Dependent Proteins* (Suttie, J. W., Ed.) pp 509−517, University Park Press, Baltimore.

Harbeck, M. D., Cheung, A. Y., & Suttie, J. W. (1989) *Thromb. Res. 56*, 317−323.
Harlow, E., & Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Hauschka, P. V., Lian, J. B., Cole, D. E., & Gundberg, C. M. (1989) *Physiol. Rev. 69*. 990−1047.
Hubbard, B. R., Jacobs, M., Ulrich, M. M. W., Walsh, C., Furie, B., & Furie, B. C. (1989) *J. Biol. Chem. 264*, 14145−14150.
Huq, N. L., Teh, L.-C., Christie, D. L., & Chapman, G. E. (1984) *Biochem. Int. 8*, 527−531.
Huq, N. L., Tseng, A., & Chapman, G. E. (1987) *Biochem. Int. 15*, 271−277.
Katow, K. J., Roth, D. A., Porter, T. J., Furie, B. C., & Furie, B. (1993) *Methods Enzymol. 222*, 435−449.
Kiefer, M. C., Saphire, A. C. A., Bauer, D. M., & Barr, P. J. (1990) *Nucleic Acids Res. 18*, 1909.
Knobloch, J. E., & Suttie, J. W. (1987) *J. Biol. Chem. 262*, 15334−15337.
Kuhn, C. C., & Crabb, J. W. (1986) in *Advanced Methods in Protein Microsequence Analysis* (Wittman-Liebold, B., Ed.) pp 65−67, Springer-Verlag, Berlin, Heidelberg.
Lee, H. H., & Miller, L. K. (1978) *J. Virol. 27*, 754−767.
Lerch, R. A., & Friesen, P. D. (1992) *J. Virol. 66*, 1590−1601.
Liska, D. J., & Suttie, J. W. (1988) *J. Biol. Chem. 27*, 8636−8641.
Lowry, O. H., Rosebrough, N. J., Farrand, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265−275.
Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035−10038.
Nishimoto, S. K., & Price, P. A. (1985) *J. Biol. Chem. 260*, 2832−2836.
Nishimoto, S. K., Araki, N., Robinson, F. D., & Waite, J. H. (1992) *J. Biol. Chem. 267*, 11600−11605.
Pharmacia Fine Chemicals (1983) *Affinity Chromatography: Principles and Methods*, pp 15−18, Piscataway, NJ.
Poser, J. W., & Price, P. A. (1979) *J. Biol. Chem. 254*, 431−436.
Poser, J. W., Esch, F. S., Ling, N. C., & Price, P. A. (1980) *J. Biol. Chem. 255*, 8685−8691.
Price, P. A. (1988) *Annu. Rev. Nutr. 8*, 565−583.
Price, P. A., & Nishimoto, S. K. (1980) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3374−3375.
Price, P. A., Poser, J. W., & Raman, N. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3374−3375.
Price, P. A., Fraser, J. D., & Metz-Virca, G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8335−8339.
Rich, D. H., Lehrman, S. R., Kawai, M., Goodman, H. L., & Suttie, J. W. (1981) *J. Med. Chem. 24*, 706−711.
Summer, M. D., & Smith, G. E. (1987) *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experiment Station Bulletin 1555, College Station, TX.
Suttie, J. W. (1993) *FASEB J. 7*, 445−452.
Ulrich, M. M. W., Furie, B., Jacobs, M. R., Vermeer, C., & Furie, B. C. (1988) *J. Biol. Chem. 263*, 9697−9702.
Wood, G. M., & Suttie, J. W. (1988) *J. Biol. Chem. 263*, 3234−3239.
Worobec, R. B., Wallace, J. H., & Huggins, C. G. (1972) *Immunochemistry 9*, 229−238.
Wu, S.-M., Soute, B. A. M., Vermeer, C., & Stafford, D. W. (1990) *J. Biol. Chem. 265*, 13124−13129.

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