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Aspartyl β-hydroxylase: *In vitro* hydroxylation of a synthetic peptide based on the structure of the first growth factor-like domain of human factor IX

(epidermal growth factor-like domain/β-hydroxyaspartic acid)

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ABSTRACT **β-Hydroxylation of aspartic acid is a post-translational modification that occurs in several vitamin K-dependent coagulation proteins. By use of a synthetic substrate comprised of the first epidermal growth factor-like domain in human factor IX and either mouse L-cell extracts or rat liver microsomes as the source of enzyme, *in vitro* aspartyl β-hydroxylation was accomplished. Aspartyl β-hydroxylase appears to require the same cofactors as known α-ketoglutarate-dependent dioxygenases. The hydroxylation reaction proceeds with the same stereospecificity and occurs only at the aspartate corresponding to the position seen *in vivo*. Further purification and characterization of this enzymatic activity should now be possible.**

Vitamin K-dependent coagulation factors VII, IX, and X and proteins C, S, and Z undergo similar posttranslational modifications including vitamin K-dependent γ-carboxylation of specific Glu residues in the N terminus (1–3) as well as β-hydroxylation of specific Asp (4, 5) and Asn (6) residues in certain epidermal growth factor (EGF)-like domains. The latter modification is independent of vitamin K as well as glutamic γ-carboxylation (7). While γ-carboxyglutamic residues mediate essential Ca^{2+}-dependent protein–phospholipid interactions (8), the functions of the β-hydroxyaspartic (βHya) and β-hydroxyasparagine residues are unknown. It has been suggested that βHya is involved in divalent cation binding in the EGF-like domains of these proteins (9–12) and that the EGF-like domains mediate specific protein–protein interactions (12–14).

Although the vitamin K-dependent glutamic γ-carboxylase has been characterized, *in vitro* Asp β-hydroxylation has not, to our knowledge, been demonstrated. A putative consensus sequence for Asx β-hydroxylation within EGF-like domains has been described: Cys-Xaa-Asx-$(Xaa)_4$-(Tyr or Phe)-Xaa-Cys-Xaa-Cys (15). With linear peptides based on the consensus sequence as potential substrates, we had looked for Asp β-hydroxylation after peptide incubation with extracts of whole cells (as well as subcellular fractions) using a variety of conditions. These preliminary studies proved uniformly unsuccessful. Reasoning that a substrate comprised of a properly folded EGF-like domain with correct disulfide bonds and containing the putative consensus sequence might (*i*) present the critical Asp to the enzyme in a more preferred conformation, (*ii*) be more resistant to extensive proteolysis during incubation, and (*iii*) be more readily recovered after incubation, we have synthesized the first EGF-like domain of human factor IX (EGF-IX_{1H}). Because this laboratory has recently shown in mammalian expression systems that βHya formation in recombinant human factor IX can be inhibited (16) either with heavy metal chelators such as 2,2′-dipyridyl (dipy) or with 2,4-pyridine dicarboxylate, an analogue of 2-ketoglutarate (KG) known to block proline hydroxylase (17), our initial experiments with EGF-IX_{1H} used experimental conditions favorable for KG dioxygenases such as prolyl or lysyl hydroxylase (18). We report here *in vitro* demonstration of β-hydroxylation of Asp residues and show that this enzymatic activity requires both Fe^{2+} and KG.

MATERIALS AND METHODS

Materials. The following reagents were obtained commercially: mouse L-cells (NCTC clone 929) (American Type Culture Collection); Dulbecco's modified Eagle's medium, L-glutamine, penicillin/streptomycin, and trypsin/EDTA (GIBCO); T150 tissue culture flasks (Costar); fetal calf serum (Flow Laboratories); 2-keto[1-^{14}C]glutaric acid ([^{14}C]KG; specific activity = 59 mCi/mmol; 1 Ci = 37 GBq) (Amersham); Protosol, Aquasol, and Econofluor-2 (New England Nuclear); bovine serum albumin (98–99%), Nonidet P-40, Tris, dithiothreitol, glutamic dehydrogenase (GDH) (EC 1.4.1.3, type II), catalase, dipy, iodoacetamide, thermolysin, tosylphenylalanine chloromethyl ketone (TPCK) trypsin, and NADH (Sigma); ferrous ammonium sulfate (Fisher); $(Pro\text{-}Pro\text{-}Gly)_{10}$ (Peninsula Laboratories); Ellman's reagent (Pierce); Sep-Pak C_{18} cartridges (Waters); *p*-methyl-butylated hydroxyanisole resin, *t*-butoxycarbonyl (Boc)-protected amino acids and all other reagents required for synthesis on the Applied Biosystems model 430 A peptide synthesizer; side-chain-protected Asp and Glu (Bachem); dithioerythritol (Chemical Dynamics, South Plainfield, NJ); *p*-cresol, *p*-thiocresol, L-ascorbic acid, and KG (Aldrich). Mouse EGF was donated by C. R. Savage (Temple University). 2-Nitro-5-thiosulfobenzoate was provided by G. Bencen (Merck).

Synthesis and Purification of EGF-IX_{1H}. Starting with 0.5 mM (0.80 g) of *p*-methyl-butylated hydroxyanisole resin (substitution at 0.62 mM of amine per g of resin), the synthesis was done in a stepwise manner using the Applied Biosystems automated peptide synthesizer (19) with reagents and conditions as described (20) for synthesizing echistatin, a disulfide-rich inhibitor of platelet aggregation. After coupling of N-terminal Asp the *t*-butoxycarbonyl group was removed with F_3CCOOH, and the peptide–resin was dried. Final weight of N-terminal-deblocked peptide–resin was 3.26 g. Half (1.6 g) of the peptide–resin was then cleaved with HF. Cleavage and subsequent oxidation of the peptide were done

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Abbreviations: EGF, epidermal growth factor; EGF-IX_{1H}, first EGF-like domain of human factor IX; βHya, β-hydroxyaspartic acid; dipy, 2,2′dipyridyl; KG, 2-ketoglutarate; [^{14}C]KG, 2-keto[1-^{14}C]glutarate; GDH, glutamic dehydrogenase.

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by using conditions as described for echistatin (20). Analytical HPLC was used to monitor the oxidation. A qualitative Ellman's test (21) was used to follow the disappearance of free sulfhydryl groups before proceeding with purification.

The crude oxidized product in solution (3 liters) was acidified by adding acetic acid (20 ml) and pumped onto a C_{18}-silica (5 × 30 cm, 15 μM, 300 Å) cartridge (Waters). The product was purified by using a manually generated step gradient. To 1 liter of a stirred 90% A buffer–10% B buffer (A = 0.1% F_3CCOOH/H_2O; B = 0.1% F_3CCOOH/CH_3CN) were added 1 liter each of the following concentrations of mobile phase in 100-ml increments: 85% A/15% B; 80% A/20% B; 76% A/24% B; 72% A/28% B; 70% A/30% B. A flow rate of 70 ml/min was used, with the product eluting at the completion of the 70% A/30% B buffer. Homogeneous fractions as determined by reverse phase-HPLC (Vydac C_1, 218TP5415) were pooled and lyophilized to give 24.7 mg of product. Purity was demonstrated by analytical HPLC. The solvents were A, 0.1% TFA/H_2O, and B, 0.1% TFA/acetonitrile. A linear gradient of 5–95% B was developed over 45 min at a flow rate of 1.5 ml/min on a Vydac C_{18} column (15 × 0.4 cm). *A* was monitored at 214 nm; a single large *A* peak with a retention time of 15.3 min was observed. The product was further characterized by amino acid analysis after hydrolysis with 6 M HCl and by automated Edman degradation (Applied Biosystems model 470A protein sequencer).

Cell Culture and Preparation of Subcellular Fractions. L-cells were cultured in Dulbecco's modified Eagle's medium containing sodium pyruvate, L-glutamine, and glucose at 1 g per liter. The medium was supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin G at 100 units/ml, and streptomycin at 100 μg/ml. Cell homogenates were prepared as described by Kao *et al.* with modifications (22). Five confluent T150 flasks were washed three times with 25 ml of phosphate-buffered saline at 4°C, and cells were scraped from the flasks. Cells were centrifuged at 100 × *g* for 5 min and resuspended in 10 mM Tris·HCl/0.2 M NaCl/0.1 M glycine/10 μM dithiothreitol/0.1% Nonidet P-40, pH 7.8 (buffer A), to a final volume of 5 × 10^7 cells per ml. The resuspended cells were then subjected to 10 strokes of a Potter–Elvehjem homogenizer at 1200 rpm. The homogenate was centrifuged at 20,000 × *g* for 30 min, and either the pellet, resuspended to an equal volume of the supernatant in buffer A, or the supernatant was used in hydroxylase assays.

Rat liver microsomes were prepared from perfused liver homogenates (2 ml of buffer per g wet weight) as described (23) and were resuspended in a volume of buffer A equal to the volume of buffer used in homogenization.

Assay for Asp β-Hydroxylation. Unless otherwise stated, the following conditions were used. All reagents were kept on ice before incubation. Incubations were done in 12 × 75 mm glass tubes in a final vol of 100 μl. Final concentrations of reagents were 50 mM Tris·HCl, pH 7.8/2 mM L-ascorbic acid/catalase at 100 μg/ml/0.1% bovine serum albumin/50 μM ferrous ammonium sulfate/10 μM dithiothreitol/125 μM [^{14}C]KG (specific activity 3 mCi/mmol). These reagents were added first to the test tubes in a vol of 25 μl followed by either 35 μl of a 0.28 mM solution of EGF-IX_{1H} or of water. After a 5-min incubation at 37°C in a shaking waterbath, the reaction was initiated by adding 40 μl of enzyme source. To recover $^{14}CO_2$ generated by decarboxylation of [^{14}C]KG during the ensuing incubations, a 1 × 3.5-cm strip of Whatman 3-mm chromatography paper presoaked in Protosol was immediately inserted into the neck of each tube, which was then capped with a 20-mm rubber-sleeve stopper. Incubation was for 40 min, unless otherwise indicated, in a 37°C shaking waterbath. All reactions were terminated by delivering 100 μl of 0.5 M KH_2PO_4 through the septum to lower pH to 5.0. Tubes were then placed on a shaker platform for 1 hr at room temperature to maximize trapping of released $^{14}CO_2$; trapping efficiency was determined to be 94%. The paper strips were then transferred to scintillation vials containing 15 ml of Econofluor-2 scintillation mixture, and the vials were counted in a Packard Tri-Carb 2000CA liquid scintillation counter (95% counting efficiency).

Recovery of Product and Identification of βHya. EGF-IX_{1H} from terminated crude assay mixtures could be adsorbed to and eluted from C_{18} Sep-Pak cartridges. The cartridges were first washed with 5 ml of 100% methanol and then equilibrated with 5 ml of water. The mixtures were loaded onto the cartridges, and unadsorbed materials were washed through with 5 ml of water. EGF-IX_{1H} was then eluted from the cartridge with 2 ml of 100% methanol, which was evaporated to dryness in a Savant Speed-Vac concentrator. The dried residue was subjected to acid hydrolysis (6 M HCl, 20 hr at 110°C, *in vacuo*) and analyzed for βHya using the cation-exchange HPLC method described earlier (13).

Overall recovery (before hydrolysis) was determined for several samples at ≈20% as follows. Dried eluates, obtained as described, from either control incubations or those containing EGF-IX_{1H} were dissolved in water, and equal aliquots were subjected to reverse-phase HPLC. Conditions were as described above, except that the column was a 30-cm-long Waters C_{18} μBondapak, and a linear gradient of 5–50% CH_3CN was developed over 30 min. Retention time of standard EGF-IX_{1H} was 23 min. Although no distinct peak at this position is seen in control eluates, one is seen in eluates from incubations containing peptide. The percent recovery of EGF-IX_{1H}, calculated using *A* values, was confirmed by quantitative sequence analysis. This recovery (20%) was then assumed for all other samples.

RESULTS AND DISCUSSION

Characterization of EGF-IX_{1H}. Fig. 1*A* shows the 39-residue peptide that was synthesized; it encompasses the first EGF-like domain of human factor IX, except that position 18 (corresponding to the partially hydroxylated Asp-64 in human factor IX) contains only Asp. Although the disulfide bonds in the EGF-like domains of human factor IX have not been determined experimentally, they have been determined in both EGF itself (24) and in an EGF-like domain of bovine factor X (25). The high homology between factors IX and X in this region strongly suggests a disulfide pattern shown in Fig. 1*A* for the EGF-like domain of factor IX. The amino acid composition of EGF-IX_{1H} (Table 1) agrees with the structure shown in Fig. 1*A*. In addition, automated sequence analyses of the peptide through residue 31 (unpublished data), as well as of cystine-containing fragments from a thermolysin digest (Fig. 1*B*), define the primary and secondary structure of EGF-IX_{1H} to be that shown in Fig. 1*A*.

***In Vitro* Hydroxylation of EGF-IX_{1H}.** Preliminary studies from this laboratory, using linear peptides (<11 amino acids) based on the putative hydroxylation consensus sequence (11) as substrate and rat liver homogenates as a potential source of Asp β-hydroxylation, provided no reproducible evidence for activity. In addition to the possibility that these linear peptides were intrinsically poor substrates, proteolytic degradation, endogenous levels of βHya, and/or a high variable background may have contributed to the negative results in this system. From our findings in Chinese hamster ovary cells that rough endoplasmic reticulum may be the site of Asp β-hydroxylation (26), rat liver microsomes were used as a potential source of hydroxylase activity. In addition, because we found that acid hydrolysates of extracts from mouse L-cells contained βHya, suggesting that Asp β-hydroxylase activity might be present in these cells, these extracts were also tested as a potential source of enzyme using EGF-IX_{1H} as substrate.

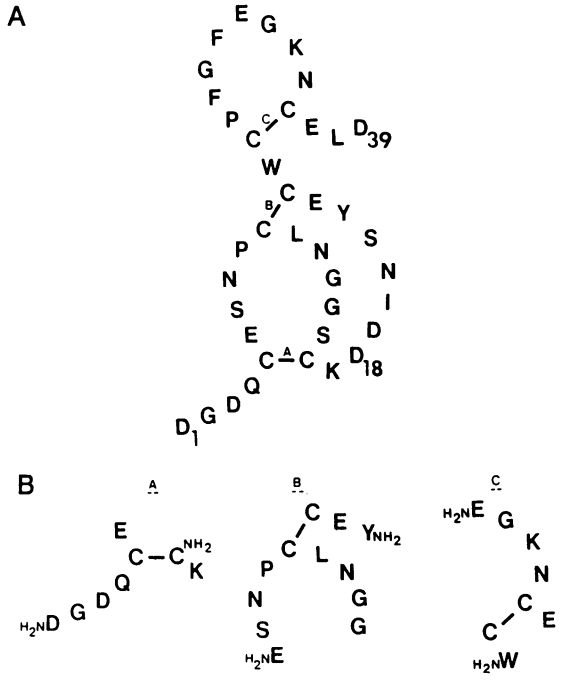

FIG. 1. Structure of EGF-IX$_{1H}$. (*A*) Amino acid sequence of EGF-IX$_{1H}$ with placement of the disulfide bonds A–C. (*B*) Fragments obtained from thermolysin digestion of EGF-IX$_{1H}$. EGF-IX$_{1H}$ (500 μg) was digested with 1 μM thermolysin (24), and the resulting peptide fragments were separated on C_{18} μBondapak (30 × 0.4 cm; Waters) column with a linear gradient of 5–50% acetonitrile developed over 30 min. Peak fractions containing cystine were sequenced.

Because our data (16) with cultured mammalian cells expressing recombinant human factor IX suggested that Asp β-hydroxylase might be a member of the Fe^{2+}/KG-dependent class of hydroxylases (i.e., prolyl or lysyl hydroxylase), we adopted incubation conditions similar to those established for enzymes of this class (18). In initial incubations of resuspended pellets or supernatants from L-cell extracts, we observed small but reproducible EGF-IX$_{1H}$-stimulated CO_2 release from KG only with the pellets. This contrasted to experiments done on the same extracts that showed that most CO_2 release stimulated by the prolyl hydroxylase substrate, (Pro-Pro-Gly)$_{10}$, occurred in the supernatant (unpublished data), as has been described (27). A significant rate of CO_2 release stimulated by EGF-IX$_{1H}$ was seen above a relative large rate independent of substrate (Fig. 2*A*). These incubation mixtures were also assayed for βHya at each timepoint (Fig. 2*B*). βHya accrued in a time-dependent manner and was readily detected only in incubations containing EGF-IX$_{1H}$. In control experiments in which EGF-IX$_{1H}$ was added after quenching the incubation, no βHya was seen. Typical HPLC tracings of control and EGF-IX$_{1H}$-containing incubations are shown in Fig. 3 *A* and *B*; significantly, the bulk of βHya is in the *erythro* form. Because the small amount of *threo*-βHya detected can be accounted for by epimerization during postincubation acid hydrolysis, our data indicate that the reaction proceeds with the same stereospecificity as seen *in vivo*. Using this highly specific and sensitive assay in incubations with supernatants, we observed no hydroxylation of EGF-IX$_{1H}$, in agreement with the CO_2-release data.

Table 1. Amino acid composition of EGF-IX$_{1H}$

Amino acid	Theoretical	Residues/mol
Alanine		
Arginine		
Aspartic acid*	9	9.26
Cysteine†	6	5.56
Glutamic acid‡	5	5.41
Glycine	5	5.07
Histidine		
Isoleucine	1	0.88
Leucine	2	1.91
Lysine	2	2.05
Methionine		
Phenylalanine	2	1.81
Proline	2	2.01
Serine	3	3.30
Threonine		
Tryptophan§	1	0.66
Tyrosine	1	0.97
Valine		

Peptide content equals 81%.
*Value includes asparagine.
†Determined as cysteic acid after performic acid oxidation.
‡Value includes glutamine.
§Determined by methane sulfonic acid hydrolysis.

Although precise determination of the stoichiometry of βHya produced to CO_2 released was not possible in this crude system, after 40-min incubation the ratio was ≈0.8 mol of βHya produced per mol of CO_2 released (Fig. 2). (This calculation assumed that the recoveries of the hydroxylated product and unhydroxylated substrate are similar.) This result suggested that Asp β-hydroxylation probably depends on KG and, furthermore, that the EGF-like substrate permits efficient coupling to decarboxylation of the putative cosubstrate. In addition, by analogy to other KG-dependent dioxygenases, these data infer an Fe^{2+} dependence as well.

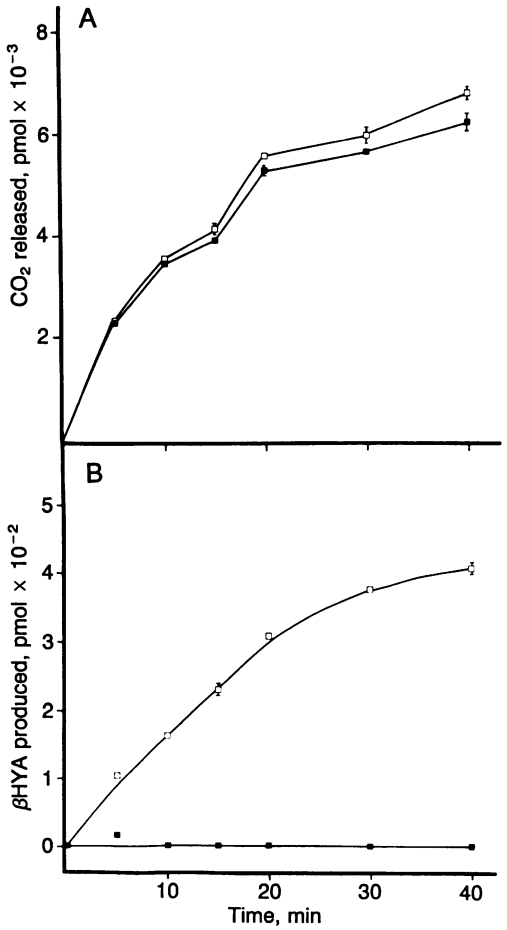

FIG. 2. Time course of EGF-IX$_{1H}$-dependent decarboxylation of KG (*A*) and βHya (βHYA) formation (*B*). L-cell pellet extracts were incubated with either H_2O (control, ■), or 1 × 10^{-4} M EGF-IX$_{1H}$ (□) for the indicated times. Values have been corrected to 100% recovery and are expressed as an average of duplicates. Error bars are shown for those duplicates having a range >4%.

To address more rigorously the putative Fe^{2+} and KG requirements, further experiments were done (Tables 2 and 3). EGF-IX_{1H}-dependent Asp β-hydroxylation can be (*i*) enhanced 6-fold by adding 50 μM Fe^{2+} and (*ii*) abolished in the presence of the heavy metal chelator dipy. The latter effect can be reversed by Fe^{2+} in molar excess of dipy. These results demonstrate that Asp β-hydroxylase has a metal ion requirement that can be satisfied by Fe^{2+}. Similarly, the results shown in Table 3 demonstrate a dependence on KG. (For the experiments in Table 3, homogenization was done in buffer A containing 0.9% Nonidet P-40. Under these conditions the supernatant from the 20,000 × *g*, 30-min centrifugation was used because 85% of hydroxylase activity was now found there; ≈50% of this activity remains in the supernatant from a further 105,000 × *g*, 1-hr centrifugation.) EGF-IX_{1H}-dependent Asp β-hydroxylation can be (*i*) enhanced 3-fold by the addition of 120 μM KG and (*ii*) inhibited by preincubation with a KG catabolizing system [GDH/NADH/$(NH_4)_2SO_4$]; this latter effect was reversed by adding KG in excess of NADH before adding substrate.

We next assayed rat liver microsomes for hydroxylase activity. Initial experiments had revealed that postincubation recoveries of EGF-IX_{1H} compared with those seen with L-cell extracts and that the background HPLC fluorescence was acceptable (see Fig. 3*C*). EGF-IX_{1H}-dependent βHya formation was detected in preparations of rat liver microsomes (Fig. 3 *C* and *D*) and also required Fe^{2+} and KG (unpublished data).

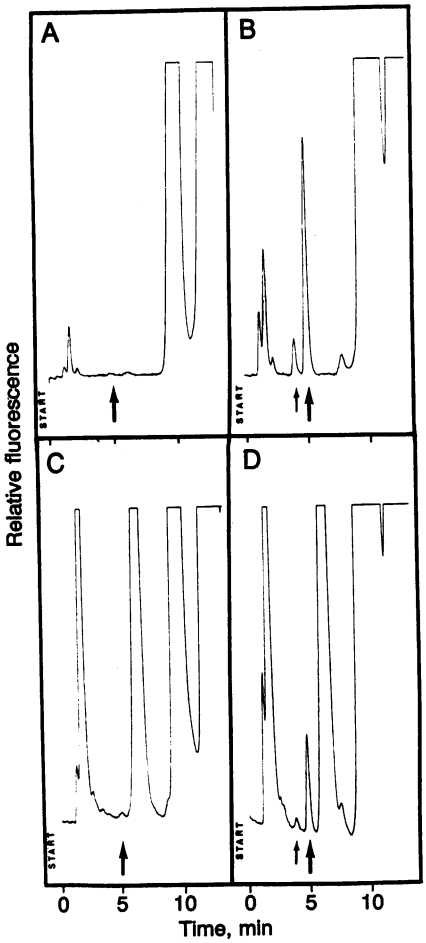

FIG. 3. HPLC of acid hydrolysates from Asp β-hydroxylation reactions. Mouse L-cell pellet extracts (*A* and *B*) or rat liver microsomes (*C* and *D*) were used as enzyme sources. H_2O (control, *A* and *C*) or 1×10^{-4} M EGF-IX_{1H} (*B* and *D*) was incubated with enzyme source for 40 min at 37°C. Large and small arrows denote the positions of *erythro*-βHya and *threo*-βHya, respectively. The larger Asp peaks (retention time, 10 min) in *B* and *D* derive from EGF-IX_{1H}.

Table 2. Effect of Fe^{2+} on Asp β-hydroxylation

Substrate	Fe^{2+}, 50 μM	dipy, 1 mM	βHya produced,* pmol
H_2O	+	−	<1
EGF-IX_{1H}	−	−	100 (±2)
EGF-IX_{1H}	+	−	591 (±109)
EGF-IX_{1H}	−	+	<1
EGF-IX_{1H}	+	+	9 (±1)
EGF-IX_{1H}	+†	+	744 (±82)

L-cell pellet extracts were incubated with (+) or without (−) dipy for 30 min at 23°C before substrate addition.
*Average of duplicates (± range).
†Ferrous ammonium sulfate (3.3 mM) was added to the dipy-treated extracts just before adding substrate.

Specificity of Hydroxylation. Because EGF-IX_{1H} was available only in small quantities and was hydroxylated to the extent of 1%, sensitivity in the 1- to 2-pmol range was required to determine the hydroxylation site within EGF-IX_{1H}. Direct identification of the phenylthiohydantoin derivative of βHya generated during Edman degradations did not satisfy this sensitivity requirement. Thus, indirect subtractive methods were used. EGF-IX_{1H} was purified from crude (hydroxylated) incubation mixtures by reverse-phase HPLC. This hydroxylated EGF-IX_{1H}, which cochromatographed with nonhydroxylated EGF-IX_{1H} and contained 0.011 nmol of βHya per nmol of total EGF-IX_{1H}, was reduced, carboxyamidomethylated, and trypsin treated. The resulting tryptic digest was resolved by reverse-phase HPLC with monitoring at 214 nm; significant levels of βHya were detected in only a single peak of OD having the sequence Asp-Asp-Ile-Asn-Ser-Tyr-Glu-Cys-Trp-Cys-Pro-Phe (DDINSYECWCPF). This peptide resulted from tryptic cleavage at the Lys-17–Asp-18 bond. (Apparent chymotryptic cleavage at the Phe-29–Gly-30 bond was also seen.) The peptide contained 0.01 nmol of βHya per nmol of peptide, which was comparable to the βHya content of hydroxylated EGF-IX_{1H} purified from the crude incubation mixtures. This result strongly suggests that the predominant site of hydroxylation occurs within this peptide, which cochromatographs with its nonhydroxylated counterpart in this system. Essentially all the βHya can be removed from the tryptic peptide after one automated Edman degradation cycle (Fig. 4). Control experiments indicate that βHya remains intact after several cycles before phenylthiohydantoin derivatization. These results define the position of Asp hydroxylation as the N-terminal Asp residue of the peptide corresponding to position 18 within the EGF-IX_{1H} sequence. The extremely low levels of βHya seen in Fig. 4*B*

Table 3. Effect of KG on Asp β-hydroxylation

Substrate	KG, 120 μM	GDH	NADH	βHya produced,* pmol
H_2O	+	−	−	<1
EGF-IX_{1H}	+	−	−	102†
EGF-IX_{1H}	−	−	−	30 (±13)
EGF-IX_{1H}	+	−	+	115 (±9)
EGF-IX_{1H}	−	+	+	4 (±4)
EGF-IX_{1H}	+	+	+	3 (±1)
EGF-IX_{1H}	+‡	+	+	106 (±13)

For these experiments homogenization was done with 0.9% Nonidet P-40. Cell supernatants from the 20,000 × *g* for 30 min centrifugation were incubated with (+) or without (−) GDH (76 units/ml), NADH (1 mM), and $(NH_4)_2SO_4$ (160 μM) for 30 min at 23°C before substrate addition.
*Average of duplicates (± range).
†Single determination.
‡KG (2.5 mM) was added to the GDH/NADH-treated supernatants just before adding substrate.

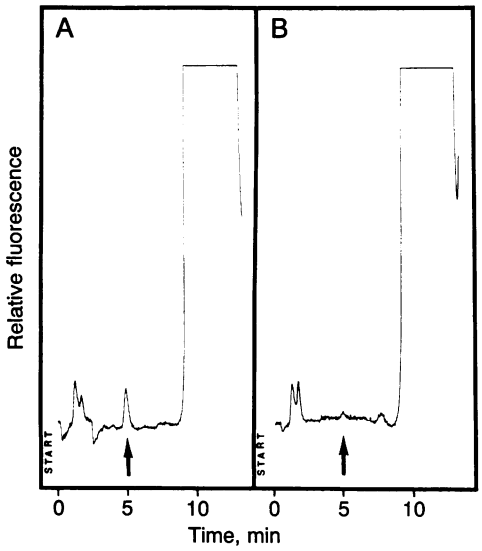

FIG. 4. βHya analysis of the single βHya-containing tryptic peptide of EGF-IX_{1H}. Hydroxylated EGF-IX_{1H} containing 0.011 nmol of βHya per nmol of peptide was purified from crude incubation mixtures by HPLC. The hydroxylated EGF-IX_{1H} (14 nmol) was then reduced and alkylated with dithiothreitol and iodoacetamide and repurified by HPLC. This derivatized peptide (10 nmol) was treated with trypsin [1:25 (wt/wt)] for 24 hr at 37°C, and the digest was then resolved by HPLC. Each resulting peptide was sequenced and analyzed for βHya. A single tryptic peptide (7 nmol) was found to contain detectable levels of βHya (0.01 nmol of βHya per nmol of peptide) and had the following sequence: Asp-Asp-Ile-Asn-Ser-Tyr-Glu-Cys-Trp-Cys-Pro-Phe (DDINSYECWCPF). Equal aliquots of this material (≈2 nmol) were applied to two individual sequence disks. One disk was washed and hydrolyzed, and the resulting hydrolysate was analyzed for βHya (*A*). The other aliquot was subjected to a single Edman degradation cycle, hydrolyzed, and the resulting hydrolysate was then analyzed for βHya (*B*). Five hundred and fifteen picomoles of peptide was recovered in *A*, containing 0.0083 nmol of βHya per nmol of peptide; and 690 pmol of peptide were recovered in *B*, containing insignificant levels of βHya.

after Edman degradation could result from either incomplete chemical coupling during the first cycle or secondary hydroxylation at Asp-19 or Asn-21.

Hydroxylation at position 18 within EGF-IX_{1H} strongly suggests that the putative consensus sequence is a requirement for hydroxylation of EGF-IX_{1H} by the crude Asp β-hydroxylase. This idea is further supported by studies with mouse EGF. Mouse EGF, which lacks the putative consensus sequence, possesses an homologous disulfide bonding pattern to EGF-IX_{1H} (24) containing a single Asp residue in each of the three disulfide loops and a single Asp residue within the C-terminal linear region. Under conditions in which EGF-IX_{1H} was hydroxylated 45 times above the limits of detectability, no βHya was detected when mouse EGF served as a substrate. (Both intact mouse EGF and EGF-IX_{1H} can be isolated from crude hydroxylation incubation mixtures with similar yields as determined by HPLC, indicating that mouse EGF is not preferentially degraded. However, the possibility that hydroxylated mouse EGF is formed and is preferentially degraded relative to hydroxylated EGF-IX_{1H} cannot be rigorously excluded.)

In summary, we have demonstrated *in vitro* Asp β-hydroxylation of the peptide EGF-IX_{1H}. Hydroxylation, which under our assay conditions has both KG and metal requirements (the latter satisfied by Fe^{2+}) and which is found in microsomal fractions, occurs predominantly, if not exclusively, at the predicted Asp within a putative hydroxylation consensus sequence with retention of the stereospecificity observed *in vivo*. While the precise molar stoichiometry of βHya formed to KG decarboxylated awaits studies with less crude preparations, our data suggest that the following equation describes the overall hydroxylation reaction:

$$\text{Asp-peptide} + O_2 + \text{KG} \xrightarrow{\text{enzyme } (Fe^{2+})} \beta\text{Hya-peptide} + CO_2 + \text{succinate.}$$

Development of this assay should allow further purification and characterization of Asp β-hydroxylase, including elucidation of its relationship to KG dioxygenases such as lysyl hydroxylase, prolyl 3-hydroxylase, and prolyl-4-hydroxylase, as well as its relationship to Asn β-hydroxylation.

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