Partial Purification and Characterization of Bovine Liver Aspartyl β-Hydroxylase*

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In vitro hydroxylation of aspartic acid has recently been demonstrated in a synthetic peptide based on the structure of the first epidermal growth factor domain in human factor IX (Gronke, R. S., VanDusen, W. J., Garsky, V. M., Jacobs, J. W., Sardana, M. K., Stern, A. M., and Friedman, P. A. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3609-3613). The putative enzyme responsible for the posttranslational modification, aspartyl β-hydroxylase, has been shown to be a member of a class of 2-ketoglutarate-dependent dioxygenases, which include prolyl-4- and lysyl-hydroxylases. In the present study, we describe the solubilization with nonionic detergent of the enzyme from bovine liver microsomes and its purification using DEAE-cellulose followed by heparin-Sepharose. No additional detergent was required during purification. The partially purified enzyme preparation was found to contain no prolyl-4- or lysyl-hydroxylase activity. Using a synthetic peptide based on the structure of the epidermal growth factor-like region in human factor X as substrate, the apparent Km values for iron and α-ketoglutarate were 3 and 5 μM, respectively. The enzyme hydroxylated the factor X peptide with the same stereospecificity (erythro β-hydroxyaspartic acid) and occurred only at the aspartate corresponding to the position seen in vivo. Furthermore, the extent to which either peptide (factor IX or X) was hydroxylated reflected the extent of hydroxylation observed for both human plasma factors IX and X.

Several vitamin K-dependent proteins (coagulation factors VII, IX, and X, as well as proteins C, S, and Z) undergo an enzymatic posttranslational modification in which a specific aspartic acid residue, located in the first growth factor-like domain of the protein, is hydroxylated on the β-carbon (1-4). In addition, β-hydroxylation of a specific asparagine residue in each of the other EGF-like domains of protein S (5) and in EGF-like domains of a number of other proteins, including uromodulin, thrombomodulin, and the complement proteins Clr and Cls, has been observed (6, 7). No definitive function has been ascribed to either hydroxylated residue although it has been suggested that they could be involved in divalent cation and/or protein-protein interactions (3, 5, 6, 8-11).

We have recently shown (12, 13) that the enzyme-catalyzing hydroxylation of aspartic acid, aspartyl β-hydroxylase (Asp β-hydroxylase), is a 2-ketoglutarate (KG)-dependent dioxygenase. Other such dioxygenases include the collagen-modifying enzymes prolyl-4- and prollyl-3-, and lysyl-hydroxylases, the t-carnitine synthesis enzymes E-N-trimethyl-lysine and γ-butyrobetaine hydroxylases as well as thymine-7-, pyrimidine deoxyribonucleoside-2-, and deoxypyridine 1'-hydroxylases (14). As with the other KG dioxygenases Asp β-hydroxylase has absolute requirements for Fe2+ and KG (13). Preliminary data suggest that like prolyl-4- and lysyl-hydroxylases (15, 16), it is located within the rough endoplasmic reticulum (17).

Our initial demonstration (13) of in vitro Asp β-hydroxylase was made with crude extracts of either mouse L-cells or rat liver microsomes using a 39-amino acid peptide substrate, based on the structure of the first EGF-like domain of human factor IX (EGF-IXml). Stenflo and coworkers (18), using a similar peptide, have reported peptide-dependent decarboxylation of KG with rat liver microsomes. Using an improved substrate, based on the structure of the first EGF-like domain of human factor X (EGF-Xnl), we now report the solubilization, partial purification, and initial characterization of bovine hepatic microsomal Asp β-hydroxylase.

EXPERIMENTAL PROCEDURES

Materials—The following reagents were obtained commercially: heparin-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.); 2-ketoglutaric acid (specific activity = 59 mCi/mmol) (American Corp.); DEAE-cellulose (DE52) (Whatman Biosystems Ltd.); Econofluor-2 (Aquafluor, and Protosol (Du Pont-New England Nuclear); [Pro-Pro-Gly]4, and lysine hydroxylase substrate (L-1) (Peninsula Laboratories); endoproteinase Glu-C (Protein V8) from Staphylococcus aureus V8 (Boehringer Mannheim); Sep Pck C6, cartridge (Waters Associates); Ultrafree-MC Filter Units (10,000 molecular weight cut-off) (Millipore); ferric ammonium sulfate, zinc chloride, and sucrose (Fisher); t-ascorbic acid, KG, 2,4-pyridine dicarboxylic acid (Aldrich Chemical Co); bovine serum albumin (98-99%), Nonidet P-40, iodoacetamide, trypsin, Tris, 2,2'-dipyridyl, and DOPA, 3-(3-cholamidopropyl)dimethylammonio)1-propanesulfonate.
Pipes, diethanolamine, catalase, CHAPS, soybean trypsin inhibitor, and Dulbecco's phosphate-buffered saline (Sigma).

Methods—EGF-XI\textsubscript{n} was chemically synthesized as described for EGF-X\textsubscript{I\textsubscript{n}} (13, 19). Position 18, which would be \(\beta\)-hydroxysaspartic acid (Hya) in mature factor X, contained only aspartic acid. The product was characterized by amino acid analysis after hydrolysis with 6 N HCl and by automated Edman degradation using a gas phase sequencer (Applied Biosystems model 470A protein sequencer).

Partial Purification of Bovine Aspartyl \(\beta\)-Hydroxylase—At a local abattoir, 300 g of fresh bovine liver was rapidly sliced and the strips immersed immediately in ice-cold Dulbecco's phosphate-buffered saline before transport to the laboratory. All procedures were carried out at 4 °C unless otherwise indicated. The tissue was minced with scissors and homogenized at top speed for 1 min in a Waring blender in 600 ml of buffer containing 20 mM Tris, 0.2 M NaCl, 0.1% glycine, and 1% Nonidet P-40, pH 7.5, using 10 strokes of a Dounce homogenizer (Wheaton Type B pestle); after 60 min the homogenate was centrifuged at 105,000 \(\times\) g for 75 min. The microsomal pellet was then gently homogenized in 300 ml of buffer containing 10 mM Tris, 0.2 M NaCl, 0.1% glycine, and 1% Nonidet P-40, pH 7.5, using 10 strokes of a Dounce homogenizer (Wheaton Type B pestle); after 60 min the homogenate was centrifuged at 105,000 \(\times\) g for 75 min. The resulting supernatant (300 ml) was diluted with 900 ml of 50 mM Tris, pH 7.5, and applied to a 1.6 \texttimes\texttimes 22-cm DE52 column, that had been equilibrated with 50 mM Tris, 50 mM NaCl, pH 7.5. The column was washed with this buffer (500 ml) until the absorbance at 280 nm dropped below 0.04 absorbance units and then the bound protein was eluted with a linear gradient formed with 250 ml of the equilibration buffer and 250 ml of 0.8 M NaCl, 50 mM Tris, pH 7.5. Flow rate throughout was 3 ml/min, and 6-ml fractions were collected. Peak elution fractions of Asp-\(\beta\)-hydroxylase activity were pooled, diluted with 3 volumes of 50 mM Tris, pH 7.5, and loaded onto a 1.6 \times 7 cm heparin-Sepharose column (flow rate = 3 ml/min) that had been equilibrated with the same buffer used to equilibrate the DE52 column. After washing with 260 ml of the buffer, the column was eluted with a linear gradient formed with 200 ml of this buffer and 200 ml of 0.75 M NaCl, 50 mM Tris, pH 7.5. Six-ml fractions were collected, and those containing the bulk of Asp-\(\beta\)-hydroxylase activity were pooled, frozen in a dry ice/ethanol bath, and stored at −70 °C for use in subsequent enzymatic assays.

For enzyme activity determinations in which either detergent-treated microsomes, the 105,000 \(\times\) g detergent-treated microsomal supernatant, or the DEAE-cellulose fractions were assayed, recovery of hydroxylated product and identification of Hya were performed using the C\textsubscript{4} Sep-Pak cation exchange high performance liquid chromatography (HPLC) method as described previously (13). For assays employing heparin-Sepharose-purified enzyme, the identification of Hya was determined by cation exchange HPLC (C\textsubscript{4} Sep Pak omitted) following direct acid hydrolysis of the assay mixture (6) and filtration through an Ultrafree-MC filtration unit. The conditions for the Asp-\(\beta\)-hydroxylation assays employed for the partial purification were as described previously (13) except that EGF-X\textsubscript{I\textsubscript{n}} was substituted for EGF-X\textsubscript{I\textsubscript{n}}. Briefly, incubations were carried out for 40 min at 37 °C in a shaking water bath in a final volume of 100 ml of either 50 mM Tris-HCl, pH 7.8, or Pipes, pH 7.0. The final concentrations of reactants were 2 mM l-aspartic acid, 100 \(\mu\)g/ml catalase, 0.1% bovine serum albumin, 50 \(\mu\)M ferrous ammonium sulfate, 10 \(\mu\)M diethanolamine, 135 \(\mu\)M [\(\text{[14C]}\)KG (specific activity 7.3 mCi/mmol), 60 \(\mu\)M RTF-X\textsubscript{I\textsubscript{n}} (unless otherwise indicated), 0.124 mg/ml enzyme protein (unless otherwise indicated). For prolyl-\(\text{\text{4-hydroxy-}}\)hydroxylase activity determinations, the substrate (Pro-Pro-Gly)\textsubscript{I\textsubscript{n}} (20) was added to a final concentration of 130 \(\mu\)M to incubations lacking EGF-XI\textsubscript{n}. Conditions were otherwise as described above. Similarly, for Fig. 2. HPLC elution profile of a combined tryptic and Glu-C digest of synthetic EGF-X\textsubscript{I\textsubscript{n}}. The tryptic (2.5 mg) and Glu-C (2.5 mg) fragments were added to 50 \(\mu\)g of EGF-X\textsubscript{I\textsubscript{n}} in 50 \(\mu\)l of 50 mM ammonium bicarbonate buffer, pH 7.9, and incubated at 37 °C for 24 h. The digest was resolved on a microbore HPLC (Applied Biosystems model 130A) using a C\textsubscript{4} column (2.1 \times 30 mm). The peptides were eluted at a flow rate of 150 \(\mu\)l/min over 50 min with a linear gradient from buffer A (0.1% trifluoroacetic acid in H\textsubscript{2}O) to 25% buffer B (acetonitrile/0.09% trifluoroacetic acid). The peak that eluted at 4.05 min was the tripeptide Gly-Phe-Glu (residues 30–32 of EGF-X\textsubscript{I\textsubscript{n}}) while the peak that eluted at 10.12 min was the dipeptide Leu-Phe (residues 38 and 39 of EGF-X\textsubscript{I\textsubscript{n}}).

Fig. 3. Placement of disulfide bonds in EGF-X\textsubscript{I\textsubscript{n}}. HPLC-purified peptides were subjected to a stepwise Edman degradation in a gas-phase sequencer (model 470A equipped with an on-line PTH analyzer model 120A from Applied Biosystems, Inc.). For peak A, (Pth-Cys), was identified at cycle 5, while for peaks B and C, (Pth-Cys) was determined at cycles 4 and 5. Peak C resulted from an incomplete trypsin digestion at Lys-34. The two peptides in peak A and three peptides in peaks B and C were in equimolar quantities. The numbers in parentheses are the positions of the residues of EGF-X\textsubscript{I\textsubscript{n}} as indicated in Fig. 1.
Aspartyl β-Hydroxylase

FIG. 4. DE-52 column chromatography of Asp β-hydroxylase. Chromatographic conditions for elution of Asp β-hydroxylase activity and for the monitoring of the activity of EGF-XIH-dependent decarboxylation of [14C]KG were as described under "Methods." Fractions between the arrows were pooled for further purification. ○, absorbance at 280 nm; □, EGF-XIH-dependent decarboxylation of [14C]KG. Protein which passed through the column during loading and initial washing (1625 ml) was collected in batches, and absorbance at 280 nm and protein (■) (29) were determined. After the first 250 ml of wash had been applied, collection and monitoring of individual fractions were begun.

![Chromatogram](image)

FIG. 5. Heparin-Sepharose column chromatography of Asp β-hydroxylase. The elution of Asp β-hydroxylase activity was as described under "Methods." ○, absorbance at 280 nm; □, EGF-XIH-dependent decarboxylation of [14C]KG; ■, formation of Hya. Protein which passed through the column during loading and initial washing (520 ml) was collected in batches, and absorbance at 280 nm was determined. After the first 150 ml of wash had been applied, collection and monitoring of individual fractions were begun. Fractions between the arrows were pooled.

![Chromatogram](image)

### Table I

Partial purification of Asp β-hydroxylase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein</th>
<th>CO₂ released from KG</th>
<th>Hya produced</th>
<th>Coupling ratio</th>
<th>Yield</th>
<th>Specific activity</th>
<th>Prolyl-4-hydroxylase CO₂ released</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>µmol</td>
<td>µmol Hya/µmol CO₂</td>
<td>%</td>
<td>µmol/mg</td>
<td>Hya</td>
<td>Fold</td>
</tr>
<tr>
<td>Detergent-treated microsomes</td>
<td>5140</td>
<td>0.5 ± 0.15</td>
<td>0.8</td>
<td>1.6 ± 0.5</td>
<td>0.10</td>
<td>0.16</td>
<td>1</td>
</tr>
<tr>
<td>105,000 × g supernatant</td>
<td>2520</td>
<td>1.7 ± 0.2</td>
<td>1.5</td>
<td>0.06 ± 0.09</td>
<td>106</td>
<td>0.07</td>
<td>0.60</td>
</tr>
<tr>
<td>DEAE-cellulose pool</td>
<td>195</td>
<td>1.2 ± 0.2</td>
<td>0.85</td>
<td>0.71 ± 0.10</td>
<td>57</td>
<td>6.2</td>
<td>4.4</td>
</tr>
<tr>
<td>Heparin-Sepharose pool</td>
<td>14</td>
<td>0.47 ± 0.06</td>
<td>0.41</td>
<td>0.57 ± 0.11</td>
<td>27</td>
<td>34</td>
<td>29</td>
</tr>
</tbody>
</table>

* Protein concentration was determined using the modified Lowry assay (29).
☆ Based on Hya produced.
< Single determination.
> Activity increased during solubilization (see "Results and Discussion").
> Greater than 90% of the applied activity was detected in the column flow-through.

lysyl hydroxylase activity determinations, the substrate L-1 was added to a final concentration of 287 µM to incubations lacking EGF-XIH (21).

### RESULTS AND DISCUSSION

Structure Determination of EGF-XIH—The primary structure of the synthetic first EGF-like domain of human factor X was confirmed by automated sequence analysis of the peptide through residue 37 (Fig. 1) and by amino acid composition (data not shown). In addition fast atom bombardment spectroscopic analysis provided a parent ion with the expected molecular weight 4245 (MH⁺).

The secondary structure was determined by identifying the cycle(s) during the Edman reaction at which diPTH-cystine...
Aspartyl \( \beta \)-Hydroxylase

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CH

WC. 6. pH dependence of Asp \( \beta \)-hydroxylation. Heparin-Sepharose-purified Asp \( \beta \)-hydroxylase was incubated in either 100 mM Pipes (pH range 5.8-7.8) or 100 mM Tris, pH range 7.3-9.3, for 40 min at 37 °C. Reactions were terminated by addition of enough 0.5 M \( \text{KH}_2\text{PO}_4 \) to lower the pH to 5.0. Product was recovered by adsorption to and elution from \( \text{C}_{18} \) Sep-Pak cartridges (13) (overall recovery \( \approx 45\%-50\% \)), and Hya was determined. Each experimental point represents the average of duplicate determinations. Error bars are shown for the duplicates having a range \( \gtrsim 5\% \). ○, Pipes buffer; ●, Tris buffer.

Fig. 7. Temperature-dependent time courses of Asp \( \beta \)-hydroxylation. Reactions were carried out at either 23 (■) or 37 °C (□) for the indicated times. Each experimental point represents the average of duplicate determinations. Error bars are shown for those duplicates having a range \( \gtrsim 5\% \).

Fig. 8. Effect of enzyme concentration on Asp \( \beta \)-hydroxylation. Incubations were for 40 min at 37 °C using the indicated amounts of enzyme. Each experimental point represents the average of duplicate determinations. Error bars are shown for duplicates having a range \( \gtrsim 5\% \).

(PTH-Cys)\(_2 \) was released from peptides that had been generated from the simultaneous enzymatic cleavages of EGF X\(_{1\text{H}} \) by trypsin and endoproteinase Glu-C (22, 23). The resulting peptides were resolved by reversed phase HPLC (Fig. 2). Sequence analyses of peak A revealed a peptide that contained a single disulfide distributed between two peptides, and peak B contained two disulfides distributed among three peptides (Fig. 3). The analysis of peak A further demonstrated that (PTH-Cys)\(_2 \) appeared only at cycle 5 and that only one PTH-amino acid, Asp, was identified at cycles 1 and 3 in a molar ratio of 1:2, respectively. These data were sufficient to define one disulfide pair in EGF-X\(_{1\text{H}} \) as occurring between Cys-5 and Cys-16 (Fig. 3). Since peak B contained 2 disulfide pairs, 3 sets of two pairs were possible among Cys-10, -25, -27, and -36 contained within the three peptides (Fig. 3). (PTH-Cys)\(_2 \) was identified only at cycles 4 and 5 thereby eliminating one possible set of pairs. Cys-10-27 and Cys-25-36. The remaining two sets of pairs cannot be distinguished by sequence analysis alone. One set, however, containing the distribution of pairs Cys-10-36 and Cys-25-27 (intra-peptidyl disulfide) would result in two noncovalently linked molecules while the set containing the cystine pairs 10-25 and 27-36 would result in a single molecule containing three interlinked peptides. Elution of these peptides within a single peak (B) in equimolar quantities strongly favors the latter set of pairs (Figs. 2 and 3). Additional evidence that these three peptides are interlinked comes from sequence analysis of peak C (Figs. 2 and 3) which resulted from incomplete trypptic cleavage at Lys-34 (Fig. 1). The resulting peptides (Fig. 3) in this peak also eluted in equimolar quantities. Less than 10% cross-contamination was observed in the sequence analyses of peaks B and C. These results define the secondary structure of EGF-X\(_{1\text{H}} \) (Fig. 1). This secondary structure is analogous to that determined for mouse EGF (24) as well as for EGF-X\(_{1\text{H}} \), a substrate for Asp \( \beta \)-hydroxylase (13).

Solubilization and Partial Purification of Bovine Asp \( \beta \)-Hydroxylase—Asp \( \beta \)-hydroxylase activity can be solubilized from bovine liver microsomes using either the nonionic detergent Nonidet P-40 or the zwitterionic detergent CHAPS (data not shown). In these studies, solubility was defined as the retention of activity in the supernatant from detergent-treated microsomes after centrifugation at 105,000 \( \times \) g for 75 min. Based upon an EGF-X\(_{1\text{H}} \)-stimulated \( \text{CO}_2 \) release assay, maximal solubilized activity was attained with each detergent at 1% at 4 °C. Since Nonidet P-40 solubilized approximately 60% more activity than CHAPS (data not shown), Nonidet P-40 was used thereafter.

The Nonidet P-40-solubilized microsomal supernatant was further purified, first by DEAE-cellulose column chromatography (Fig. 4, “Methods”) followed by heparin-Sepharose column chromatography (Fig. 5, “Methods”). For the DEAE-cellulose step, 90% of the total protein passed through the column under the loading conditions while no Asp \( \beta \)-hydroxylase activity was detected in the flow-through. The DEAE-elution profile consisted of two large absorbance (280 nm) peaks, the first of which contained 80% of the bound protein and 90% of the Asp \( \beta \)-hydroxylase activity. The peak of activity eluted at about 0.2 M \( \text{NaCl} \), and greater than 90% of the total activity applied was recovered. The peak activity fractions were pooled resulting in a 7-8-fold purification at
FIG. 9. Effects of KG and Fe^{2+} concentrations on Asp \( \beta \)-hydroxylation. Each experimental point represents the average of duplicate determinations. Error bars are shown for duplicates having a range of >5%. Final concentrations of components were as described under "Methods" except the final volume was 50 \( \mu \)l. A, the initial concentration of KG in the incubations was varied as indicated. The initial Fe^{2+} concentration was 50 \( \mu \)M. Inset, double reciprocal plot of the data. B, the initial concentration of Fe^{2+} in the incubations was varied as indicated. The initial KG concentration was 125 \( \mu \)M. Inset, double-reciprocal plot of the data. In the absence of added Fe^{2+}, the reaction velocity was equal to 0.19 \( V_{\text{max}} \). This rate could be reduced to zero by the addition of 1 mM 2,2'-dipyridyl (0 Fe^{2+}). The other values were uncorrected for endogenous Fe^{2+} which from these data were estimated to be 1 \( \mu \)M.

TABLE II

Effects of PDCA, Zn^{2+}, and ascorbate on Asp \( \beta \)-hydroxylase

<table>
<thead>
<tr>
<th>Condition</th>
<th>Activity* (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>300 ± 2</td>
</tr>
<tr>
<td>+ 2 ( \mu )M PDCA</td>
<td>160 ± 3</td>
</tr>
<tr>
<td>+ 20 ( \mu )M PDCA</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>+ 200 ( \mu )M PDCA</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>+ 0.6 ( \mu )M Zn^{2+}</td>
<td>106 ± 1</td>
</tr>
<tr>
<td>+ 6 ( \mu )M Zn^{2+}</td>
<td>160 ± 1</td>
</tr>
<tr>
<td>Control minus ascorbate</td>
<td>92 ± 5</td>
</tr>
</tbody>
</table>

*Average of duplicates (±range).

Asp \( \beta \)-hydroxylase assays were carried out as described under Methods. Aliquots from stock solutions of PDCA (2 mm) or ZnCl\(_2\) (6 mm) in 80 mM Pipes, pH 7.0, were added to reaction mixtures to give the concentrations indicated just prior to addition of enzyme.

TABLE I

Characterization of Partially Purified Asp \( \beta \)-Hydroxylase—

The optimal pH for the enzyme was found to be between 6.7 and 7.5 (Fig. 6). No significant difference in activity or coupling ratio was observed when 0.1 M Tris buffer was replaced by 0.1 M Pipes at pH 7.3 and 7.8. All subsequent Asp \( \beta \)-hydroxylase studies were performed at pH 7.0 in Pipes buffer.

Hya formation remained linear for 40 min at 37 °C and, by centrifugation at 105,000 \( \times \) g resulted in an overall increase in solubilized enzyme activity as monitored by either CO\(_2\) release from KG or formation of Hya (Table I). It is unclear whether this apparent inhibition by the microsomal membranes is occurring through a specific or nonspecific mechanism since an increase in stimulated CO\(_2\) release from KG by a prolyl-4-hydroxylase-specific substrate (Pro-Pro-Gly)\(_{10}\) upon the removal of the microsomal membranes was also observed (Table I). The coupling ratio, defined as the ratio of moles of Hya formed to moles of CO\(_2\) (EGF-X\(_{19}\) stimulated) released, was approximately 0.8 and remained unchanged from the 105,000 \( \times \) g supernatant step through the heparin-Sepharose step (Table I). This coupling ratio was independent of whether EGF-X\(_{19}\) or EGF-IX\(_{19}\) was used as a substrate (data not shown). The coupling ratio observed in the detergent-treated microsomal fraction, 1.6 ± 0.5, was greater than 1.0, the value that would define a totally coupled system. At this early stage of purification, it was difficult to accurately determine the amount of EGF-X\(_{19}\) stimulated CO\(_2\) released from KG since it was only 25% of the amount of CO\(_2\) released in the absence of substrate (13). Thus, the uncertainty in this determination was reflected in the value of the coupling ratio derived from it. The Asp \( \beta \)-hydroxylase activity of the heparin-Sepharose fraction was stable at −70 °C for at least several weeks.

Removal of the detergent-treated microsomal membranes this step. Detergent in the elution buffer was not required. This pool of activity was diluted and applied to a heparin-Sepharose column at pH 7.5 (Fig. 5). Approximately 70% of the total protein passed through the column; no hydroxylase activity was detected in the flow-through. The peak of hydroxylase activity eluted at about 0.5 M NaCl. The purification from solubilized microsomes through heparin-Sepharose was 180-fold (Table I) with an overall recovery from the microsomal supernatant of 27% based upon Hya. The Asp \( \beta \)-hydroxylase activity was separated from prolyl-4-hydroxylase activity on the heparin-Sepharose column (Table I) and contained no lysyl-hydroxylase activity, indicating that Asp \( \beta \)-hydroxylase is distinct from these two mammalian KG-dependent dioxygenases (20, 21).
FIG. 10. Effects of EGF-XIH and EGF-IXIH concentrations on Asp \(\beta\)-hydroxylation. Each experimental point represents the average of duplicate determinations. Error bars are shown for duplicates having a range >5%. A, EGF-XIH (■) and EGF-IXIH (▲) at the indicated concentrations were incubated with enzyme. Substrates were diluted into 50 mM Pipes buffer, pH 7.0, containing 50 mM NaCl and 0.02% bovine serum albumin. B, double-reciprocal plot of the data.

![Diagram](http://www.jbc.org/)

**FIG. 11. Identification of the site of hydroxylation of EGF-XIH.** Hydroxylated EGF-XIH (23 nmol) containing 0.086 mol of Hya/mol of peptide was purified from crude incubation mixtures by reversed phase HPLC (13). The hydroxylated EGF-XIH was reduced with dithiothreitol, alkylated with iodoacacetamide, and repurified by reversed phase HPLC (13). The derivatized peptide was digested with trypsin (15:1 (w/w)) for 8 h at 37 °C. The tryptic digestion products were fractionated via reversed phase HPLC. The solvents were A, 0.1% trifluoroacetic acid/H$_2$O, and B, 0.1% trifluoroacetic acid/acetonitrile. A linear gradient of 5-50% B was developed over 60 min at a flow rate of 1.5 ml/min on a Vydac C$_8$ column (15 × 0.4 cm). The darkened area of the elution profile represents the continuous monitoring of absorbance at 215 nm. Each fraction was analyzed for Hya. The histogram denotes the amount of Hya in each fraction, and the solid line traversing the profile shows the acetonitrile gradient. Shown below the profile are the amino acid sequences within the fractions which either contained detectable levels of Hya or an absorbance peak. (In addition to expected tryptic peptides, random cleavages were also observed.) Also indicated are the Hya contents of these fractions as determined by amino acid analysis. A dashed line indicates that no Hya was detected.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sequence</th>
<th>Hya (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF-XIH</td>
<td>DGDQCETSPCNQKCKGDGLGEYTCCTLEGFEGKNCELF</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>DGDQCETSPCNQK</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>DGDEYT</td>
<td>109.2</td>
</tr>
<tr>
<td>9</td>
<td>CKDGLEYT</td>
<td>93.2</td>
</tr>
<tr>
<td>10</td>
<td>CKDGLEYTTC</td>
<td>0.8</td>
</tr>
<tr>
<td>11</td>
<td>CLEGFEGK</td>
<td>50.8</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>TCTCLEGEGK</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
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<tr>
<td>15</td>
<td>CKDGLEYTTCLE</td>
<td>26.8</td>
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<td>190.2</td>
</tr>
<tr>
<td>18</td>
<td>CKDGLEYTTCLEGFEGK</td>
<td>150.0</td>
</tr>
<tr>
<td>19</td>
<td>CKDGLEYTTCLEGFPEG</td>
<td>16.4</td>
</tr>
</tbody>
</table>

except for a 5 min lag, also at 23 °C (Fig. 7). The amount of hydroxylated product formed at 37 °C was 1.8 times greater than the amount formed at 23 °C. Subsequent reactions were carried out for 40 min at 37 °C. Under these conditions the amount of Hya formed was not linear with respect to the concentration of enzyme used but exhibited a sigmoideal relationship (Fig. 8). The sigmoideal shape of the curve is not presently understood. Linearity of the reaction with respect to time would argue that this phenomenon does not result from a complex interaction(s) between hydroxylated product and Asp \(\beta\)-hydroxylase.

Previous results using mouse L-cell extracts and rat liver microsomes demonstrated that Asp \(\beta\) hydroxylase was absolutely dependent upon KG and had a metal ion requirement.
that could be satisfied by the addition of Fe³⁺ (13), thus classifying the enzyme as a KG-dependent dioxygenase. The apparent $K_m$ values for Fe³⁺ and KG using the partially purified enzyme and EGF-X₁₁₁ were approximately 3 and 5 μM, respectively (Fig. 9). These values are similar to those reported for rat skin and chick embryo prolyl 4-hydroxylase (25, 26). Prolyl-4-hydroxylase is inhibited by Zn²⁺ (15) as well as by the KG analogue 2,4-pyridine dicarboxylate (PDCA) (27).

In the presence of either of these, inhibition of Asp $β$-hydroxylase was observed (Table II), consistent with all previous data indicating similar mechanisms for the two enzymes. In addition, ascorbate was found to be a significant activator but not an absolute requirement for Asp $β$-hydroxylase as has been found with prolyl 4-hydroxylase (28).

Human plasma factor IX contains 0.2-0.3 mol of Hya/mol of factor IX while human plasma factor X contains 0.8-1.0 mol of Hya/mol of factor X (4). Therefore, it was of interest to determine the substrate kinetic parameters of EGF-IΧ₁₁₁ and EGF-XΙΙ using the heparin-Sepharose-purified Asp $β$-hydroxylase. Over an 80-fold concentration range (Fig. 10) EGF-XΙΙ was a significantly better substrate than EGF-IΧ₁₁₁.

Although the $K_m$ values were equivalent (30 μM) the $V_{max}/K_m$ value for EGF-XΙΙ is about four times greater than the $V_{max}/K_m$ value for EGF-IΧ₁₁₁. (The apparent substrate inhibition observed for EGF-XΙΙ would tend to lower the individual $V_{max}$ and $K_m$ values but would not significantly affect the $V_{max}/K_m$ value obtained at low substrate concentrations.) Thus, the relative EGF-IΧ₁₁₁ and EGF-XΙΙ substrate kinetic parameters correlate well with the aforementioned in vivo extents of hydroxylation of factors IX and X. It is possible that amino acid substitutions within the EGF-like domains themselves (as opposed to differences outside the EGF-like domains in the molecules, differences in clearance rates of hydroxylated versus unhydroxylated factors IX and X, etc.) can give rise to the varying extents of hydroxylation observed in vivo.

Demonstrating that the heparin-Sepharose-purified Asp $β$-hydroxylase catalyzes hydroxylation at Asp-18 in EGF-XΙΙ in vivo would provide strong evidence that this activity is the physiologically relevant enzyme. In addition, it would validate comparisons between the substrate kinetic parameters of the EGF-like domains and the extents of hydroxylation of the corresponding proteins in vivo. The site of hydroxylation was determined as described in Fig. 11. The Hya-positive fractions 16, 17, and 18 each contained a single peptide which included Asp-18 and no other Asp or Asn. Coelution of Hya-containing peptides and their unhydroxylated counterparts has been observed previously (13). In contrast, no significant Hya in the region of the peptide which contained Asp-1, Asp-3 and Asp-15, 16, 17, 18 each contained a single peptide which included Asp-1, Asp-3, Asn-12 (fraction 6) was detected. Some Hya was detected in the region preceding the elution of the peptide containing Asp-35 (fraction 11). However, the Hya in fractions 8, 9, and 10 can all be accounted for by peptides containing only Asp-18. In addition, enzymatic hydrolysis of the hydroxylated EGF-XΙΙ with Pronase followed by aminopeptidase (6), under conditions which liberate Hya as well as β-hydroxyasparagine from proteins, produced only Hya (data not shown). Thus, hydroxylation of EGF-XΙΙ by the heparin-Sepharose fraction of Asp $β$-hydroxylase occurred mostly, if not exclusively, at Asp-18 corresponding to the hydroxylation site observed in vivo for human factor X. In addition, hydroxylation within EGF-XΙΙ occurs with the same stereospecificity (erythro) as found in vivo for human factor X (4). Similar observations have been made with EGF-IΧ₁₁₁ (13).

The results of this study demonstrate that Asp $β$-hydroxylase activity can be solubilized and further purification can be achieved in the absence of additional detergent. This activity is distinct from prolyl-4-hydroxylase and lysyl-hydroxylase. When EGF-XΙΙ was substituted at the Asp-18 position with an asparagine residue, incubation with the Asp $β$-hydroxylase preparation yielded erythro-$β$-hydroxyasparagine upon enzymatic digestion. In contrast (as indicated above), under the same conditions no erythro-$β$-hydroxyasparagine was detected with the EGF-XΙΙ used in these studies. Further purification and characterization is required to resolve the relationship between the asparagine and Asp $β$-hydroxylase activities.

The correlation between the relative substrate kinetic parameters of EGF-IΧ₁₁₁ and EGF-XΙΙ and the extents of hydroxylation of human factors IX and X that occur in vivo suggests that the in vitro Asp $β$-hydroxylation system might be used to determine the amino acids and their positions within the EGF-like domains which are absolutely required for hydroxylation as well as those which can modulate the extent of hydroxylation. These results can be corroborated by site-specific mutagenesis studies in mammalian cells which express human factors IX and X. Such studies would allow one to determine the potential role(s) of conserved amino acids within the EGF-like domains in either hydroxylation recognition, (hydroxylated) EGF function, or both.

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Aspartyl β-Hydroxylase


Partial purification and characterization of bovine liver aspartyl beta-hydroxylase.


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