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INVERTEBRATE ASPARTYL/ASPARAGINYL β-HYDROXYLASE: POTENTIAL MODIFICATION OF ENDOGENOUS EPIDERMAL GROWTH FACTOR-LIKE MODULES

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Summary: An invertebrate α-ketoglutarate-dependent aspartyl/asparaginyl β-hydroxylase, which posttranslationally hydroxylates specific aspartyl or asparaginyl residues within epidermal growth factor-like modules, was identified, partially purified and characterized. Preparations derived from two insect cell lines catalyzed the hydroxylation of the expected asparaginyl residue within a synthetic epidermal growth factor-like module. This activity was found to be similar to that of the purified mammalian aspartyl/asparaginyl β-hydroxylase with respect to cofactor requirements, stereochemistry and substrate sequence specificity. Furthermore, recombinant human C1r, expressed in an insect cell-derived baculovirus expression system, was also found to be hydroxylated at the expected asparaginyl residue. Thus, these results establish the potential for invertebrate aspartyl/asparaginyl hydroxylation. Since several invertebrate proteins known to be required for proper embryonic development contain a putative consensus sequence that may be required for hydroxylation, the studies presented here provide the basis for further investigations concerned with identifying hydroxylated invertebrate proteins and determining their physiologic function.

The posttranslational β-hydroxylation of specific aspartyl (asp) and asparaginyl (asn) residues has been shown to occur stereospecifically within certain epidermal growth factor-like (EGF) modules of several mammalian proteins (1-5). Although these modules have been shown to bind calcium (1, 6-9) and to be involved in functional protein-protein interactions (1), the role of the hydroxylation itself has not been defined. A comparison of primary structures within these hydroxylated modules has suggested a consensus sequence

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Abbreviations: asp, aspartyl; asn, asparaginyl; eHya, erythro β-hydroxyaspartic acid; EGF, epidermal growth factor; EGF-S_{2B}, peptide whose structure is based on the second EGF module of bovine Protein S; rhC1r, activated recombinant human C1r; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; STI, soybean trypsin inhibitor; DTT, dithiothreitol; NP-40, Nonidet P-40; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis; IgG, immunoglobulin G; TFA, trifluoroacetic acid.

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(CXD/NXXXXF/YXCXC) that may be required for hydroxylation (1, 3, 4). Invertebrate proteins, such as Lin-12 of *Caenorhabditis elegans* and Notch of *Drosophila melanogaster*, which are required for proper embryonic development (10, 11), have also been found to contain this sequence within their EGF modules (10-16). Since these proteins are in low abundance, it is not known whether they are hydroxylated. To establish the potential for asp/asn hydroxylation in invertebrates and thereby identify systems that could prove useful for studying the biologic role of hydroxylation, we determined whether two invertebrate cell lines derived from *Drosophila melanogaster* and *Spodoptera frugiperda* possess hydroxylase activity. We have identified asp/asn hydroxylase activity in both of these cell lines. This activity is similar to that of the bovine enzyme (17-19) with respect to cofactor requirements, stereospecificity and substrate sequence specificity.

Experimental Procedures: *Partial Purification of Invertebrate Hydroxylase*-- Sf9 cells (ATCC CRL 1711) were grown at 27°C, ambient atmosphere in serum-free, low-protein medium (EX-CELL 400, JRH Biosciences). S2 cells, a gift from Dr. M. Young (Rockefeller Univ., NY), were grown at 23°C, ambient atmosphere in Schneider's medium (Gibco) containing 100 U Penicillin G/mL, 0.1 mg Streptomycin/mL (Sigma), and 13% fetal bovine serum (BioWhittaker). All the following steps were performed at 4°C. Cell extracts were prepared by washing the cells (4×10^9) 3 times in 50 mLs of PBS (Sigma) containing 1 μg/ml leupeptin and 1.0 mM PMSF, resuspending them (7.5×10^7 cells/mL) in buffer containing 10 mM Tris-Cl, pH 7.8, 0.2 M NaCl, 10 uM DTT, 0.9% NP-40, 1 μg/mL each of leupeptin, aprotinin, and STI, 10 μg/mL of benzamidine and 1.0 mM PMSF, followed by sonication for 1 min on ice. Soluble protein, obtained by centrifugation of the lysed cells at 105,000 g for 1 hour, was diluted with an equal volume of a buffer containing 20 mM Tris-Cl, pH 7.5, 50 mM NaCl, 0.9% NP-40 and the above inhibitors (buffer B), and then applied with a flow rate of 1 mL/min to a 20 mL heparin-Sepharose column (1.6 x 10 cm, Pharmacia) equilibrated in buffer B. The column was washed with 40 mLs of buffer B and step-eluted with 30 mLs each of buffer B containing final concentrations of 0.2 M NaCl, 0.5 M NaCl and 1.0 M NaCl. The elution fractions were concentrated 10-fold using Centricon 30 microconcentrators (Amicon) and then assayed. The 0.5 M NaCl elution fraction, having the highest specific activity and containing 75-85% of the total activity, was used. Typical specific activities were: Sf9, 35 pmol CO_2/min/mg protein at 23°C; S2, 25 pmol CO_2/min/mg protein at 23°C.

Hydroxylase activity assays-- Assays for $^{14}CO_2$ release from α-keto [1-^{14}C] glutaric acid and eHya formation were performed in duplicate as previously described (17) using 30 μL reaction mixtures incubated at 23°C, pH 7.4, in the presence and absence of 55 uM EGF-S_{2B}, unless otherwise indicated. Assays were initiated by the addition of enzyme (Sf9 (144 μg in 12 μL) or S2 (115 μg in 12 μL)) and quenched with 45 μL of 0.5 M KH_2PO_4. The amount of eHya formed at 40 min of incubation was directly proportional to cell extract protein concentration (0 to 144 μg Sf9 protein, 0 to 115 μg S2 protein, data not shown). The peptide substrate, EGF-S_{2B}, was synthesized using methods previously described (17). The primary structure was confirmed by sequence analysis (Figure 2B); the disulfide bonding pattern has not been determined. The retention time of EGF-S_{2B} in a previously described system (17) is 19 min.

Purification and Characterization of Hydroxylated Reaction Product-- Partially hydroxylated EGF-S_{2B} was isolated from assay mixtures by reverse phase HPLC and analyzed for eHya as previously described (17). The position of hydroxylation was determined as follows: HPLC-isolated EGF-S_{2B} was reduced and alkylated as described (17), lyophilized and resuspended to 1 mg peptide/mL in 100 mM NH_4HCO_3, pH 7.8. Endoproteinase Glu-C (Protein V8) from staphylococcus aureus V8 (Boehringer Mannheim) was added (1:25 (w/w)) for 24 hours at 37°C. The resulting digest was resolved by HPLC as described by Gronke, et al. (17) and the eHya content and sequence of the resolved peptides were determined.

Purification and Characterization of Recombinant Human C1r-- The cloning and expression of human C1r in an Sf9 baculovirus expression system was performed as previously

described (20). However, under our cell culture conditions, the C1r was expressed in its activated form (rhC1$\bar{r}$). Recombinant hC1$\bar{r}$ was isolated from expression medium in which Sf9 cells were infected with recombinant virus stock (20) and grown to approximately 1 x 10^6 cells/mL. Expression medium (250 mLs) was adjusted to pH 7.0 by the addition of NaOH, filtered through a 0.2 micron filter, and applied with a flow rate of 1 mL/min at 4°C to an immunoaffinity column (1 x 2.5 cm) consisting of immobilized sheep antihuman C1$\bar{r}$ IgG, prepared as follows. Sheep antihuman C1$\bar{r}$ IgG was purified by applying sheep antiserum (The Binding Site) to immobilized human C1$\bar{r}$ (Enzyme Research Laboratories); 2 mLs of 1 mg C1$\bar{r}$/mL resin were prepared with CNBr-activated Sepharose according to manufacturer's instructions, and the resin (1 x 2.5 cm) was equilibrated at 4°C with 25 mM Na_2HPO_4, pH 7.0, 0.5 M NaCl. Following a PBS wash, elution of IgG was achieved with 100 mM glycine, pH 2.2. Following neutralization with 1 M Tris base, the fractions containing IgG were dialyzed against 100 mM NH_4HCO_3, pH 8.3, 0.5 M NaCl (25 mL of antiserum yielded 5 mg IgG). The IgG was then immobilized to Sepharose (2 mLs of 3 mg IgG/mL resin) as described and equilibrated at 4°C with 50 mM Tris, pH 7.5, 0.5 M NaCl (equilibration buffer). Following application of expression medium, the column was washed at room temperature with equilibration buffer, and rhC1$\bar{r}$ was eluted with 20 mM Tris, pH 7.5 containing 3 M NaSCN, then dialyzed against 100 mM NH_4HCO_3. Approximately 50 ug of rhC1$\bar{r}$ were routinely obtained from 250 mLs of expression medium. SDS-PAGE (15% acrylamide, non-reducing conditions) indicated that in addition to a band corresponding to intact rhC1$\bar{r}$, there were several other bands present following immunoaffinity purification. All were immunoreactive with anti-hC1$\bar{r}$ antiserum, suggesting that they were rhC1$\bar{r}$-derived material. Expression medium derived from non-infected Sf9 cells did not contain any immunoreactive material. In experiment I (see Figure 3), rhC1$\bar{r}$ was further purified by HPLC on a C4 column (0.54 x 15 cm, Vydac) equilibrated with 20% (v/v) acetonitrile in aqueous 0.1% TFA. The column was developed with a linear gradient of 20 to 70% acetonitrile over 45 min at 1.0 mL/min. In experiment II, immunoaffinity purified rhC1$\bar{r}$ was reduced with DTT and alkylated with iodoacetamide as described (21), and applied to 2 size exclusion HPLC columns (Waters, Protein Pak SW 300, 0.75 x 30 cm), connected in series. The solvent was 6 M urea, 0.2 M formic acid, flow rate = 0.5 mL/min. The A chain was found to be >90% pure by SDS-PAGE (silver stain) and sequence analyses. The B chain was further purified to apparent homogeneity by C4 HPLC as described above. In experiment III, A chain was obtained as just described and cleaved with formic acid (70%, v/v) for 48 hours at 40°C. Equimolar portions (101 pmol Hya) of the formic acid cleavage mixture were applied to two sequencing discs. The discs were subjected to 10 or 11 cycles of Edman degradation, respectively, hydrolyzed and analyzed for eHya and total peptide.

Results and Discussion: α-Ketoglutarate-dependent hydroxylase activity was demonstrated (Figure 1, Table 1) in partially purified preparations of Sf9 (a cell line of the moth *Spodoptera frugiperda* used frequently in baculovirus expression systems (20)) and S2 (a cultured *Drosophila melanogaster* cell line) cell extracts using a synthetic peptide whose primary structure is based upon the second EGF module of bovine Protein S (3) (EGF-S_{2B}; see Figure 2B for primary structure). Initial screening of EGF substrates used in mammalian studies (18, 19) indicated that EGF-S_{2B} was significantly preferred over the others tested in these two invertebrate systems (see below). Analyses of progress curves indicated that EGF-S_{2B}-dependent CO_2 release from α-ketoglutarate (A and B, Figure 1) paralleled hydroxylation of EGF-S_{2B} as monitored by eHya formation (C and D, Figure 1). The stereochemistry and ratio of eHya to CO_2 released (coupling ratio ≅ 0.6 - 0.9, Figure 1, Table 1) were similar to what was observed with the bovine liver asp/asn hydroxylase (18, 19). In accord with these results, eHya formation was dependent upon α-ketoglutarate (Table 1), indicating that the invertebrate hydroxylases, like their mammalian counterpart (18, 19, 22), are α-ketoglutarate-dependent dioxygenases. These invertebrate activities also possessed a metal ion requirement that could be satisfied by Fe^{2+}, and were inhibited by general α-ketoglutarate-dependent dioxygenase inhibitors (Table 1).

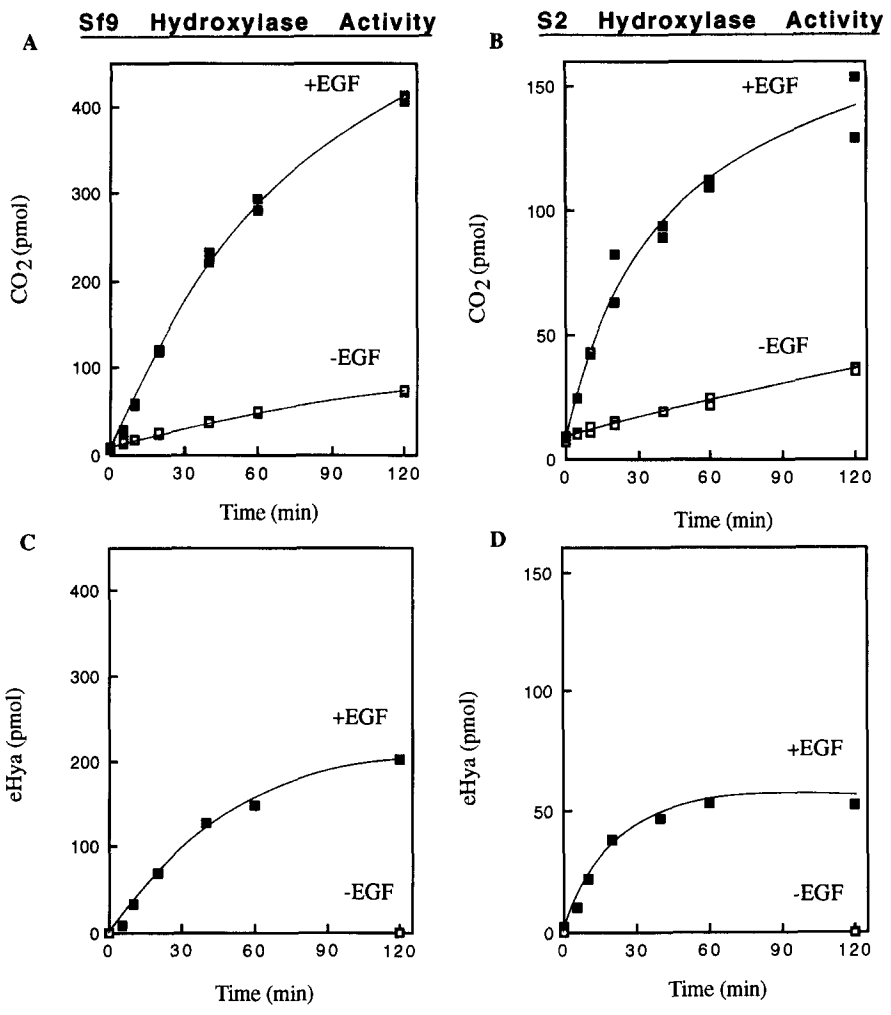


Figure 1. EGF-S_{2B}-stimulated release of CO_2 from α-ketoglutarate and asn hydroxylation of EGF-S_{2B} by extracts of Sf9 and S2 cells. Assays for $^{14}CO_2$ release (A and B) and eHya formation (C and D) were performed as described in Experimental Procedures. Assays were quenched at the indicated times. One assay mixture per time point was analyzed for eHya. erythro β-hydroxyasparagine is converted to eHya during acid hydrolysis used in this analysis (3, 4). The amount of threo β-hydroxyaspartic acid generated (5-10% of total Hya, data not shown) was that amount expected from pure eHya that has undergone epimerization during analysis. The coupling ratio is defined as mol eHya/mol CO_2, where mol CO_2 is determined by subtracting the amount of EGF-S_{2B}-independent CO_2 (❑) from EGF-S_{2B}-dependent CO_2 (■).

Although the invertebrate enzymes were similar to the mammalian enzyme with respect to cofactor requirements and stereospecificity, it was necessary to determine whether they hydroxylated the expected asn residue (asn 21) within the EGF-S_{2B} consensus sequence. Partially hydroxylated EGF-S_{2B} (0.13 mol eHya/mol peptide) was isolated from Sf9 reaction mixtures, derivatized and subsequently digested with endoproteinase Glu-C as described in Experimental Procedures; the digest was then resolved by HPLC (Figure 2A). A peptide including asn 21 was found in each eHya-containing peak (Figure 2B). In each of these peaks, the ratio of eHya to asn 21-containing peptide was constant and equal to that of hydroxylated

Table 1 Cofactor requirements of Sf9 and S2 hydroxylase activities

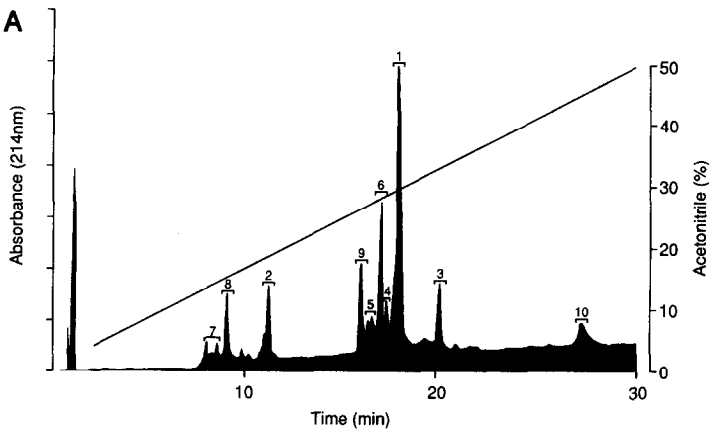
	CO_2 (pmol)	eHya (pmol)	%Control
Sf9 control	218 ± 9	204 ± 7	100
-Fe^{2+}	24 ± 11	21 ± 10	10
- Fe^{2+}, +100μM EDTA	2 ± 1	≤2	≤1
-αKG	----	4 ± 1	2
+ 500 μM PDCA	2 ± 1	≤2	≤1
+ 500 μM DIPY	1 ± 1	≤2	≤1
S2 control	87 ± 4	56 ± 5	100
-Fe^{2+}	16 ± 1	10 ± 2	18
-Fe^{2+}, +100 μM EDTA	2 ± 2	≤2	≤4
-αKG	----	≤2	≤4
+ 500 μM PDCA	2 ± 1	≤2	≤4
+ 500 μM DIPY	1 ± 1	≤2	≤4

Assays were performed as described in Experimental Procedures with omissions (-) and additions (+) as indicated. Control assays contained all the required components. The conditions for Sf9 hydroxylase assays were: 40 min at 37°C, 78 μM EGF-S_{2B}. The conditions for S2 hydroxylase assays were: 60 min at 23°C, 55 μM EGF-S_{2B}. Inhibitors (EDTA, ethylenediaminetetraacetic acid; PDCA, 2,4-pyridinedicarboxylic acid; DIPY, 2,2'-dipyridyl), dissolved in assay buffer, were added immediately prior to addition of enzyme. Means of duplicates ± range are indicated. eHya data were used to calculate %control values. The limit of sensitivity is ≤ 2 pmol eHya.

EGF-S_{2B}, indicating that these peptides cochromatographed with their nonhydroxylated counterparts (17, 18). Furthermore, peaks 8 and 9 (4700 and 1800 pmoles peptide, respectively) lacked asn 21 and had no detectable eHya, suggesting that significant hydroxylation had not occurred within these regions of EGF-S_{2B}. When peak 1, which contained the most eHya as well as a peptide which had asn 21 at its amino terminus, was subjected to one cycle of Edman degradation (Figure 2B), 98% of the eHya was removed when compared to a 0 cycle control sample. A control experiment with intact, partially hydroxylated, derivatized EGF-S_{2B} indicated that eHya was stable to Edman degradation. These data indicate that hydroxylation had occurred at the expected position of EGF-S_{2B}.

The ability of an Sf9 cell extract to hydroxylate a small peptide strongly suggests that some endogenous invertebrate proteins are hydroxylated. However, the proteins known to contain the consensus sequence within EGF modules are present in very low abundance, and a technique to determine low (≤ 2 pmol) levels of eHya in crude fractions does not exist. Therefore, we determined whether Sf9 cells, when used in a baculovirus expression system, could hydroxylate a mammalian protein known to be endogenously hydroxylated. Recombinant human C1r, isolated in its activated form (rhC1r̄), was purified (>90%) by immunoaffinity chromatography followed by C4 HPLC and was found to be hydroxylated to approximately 8% (Figure 3, experiment I). In a second experiment, a comparable preparation of immunoaffinity purified rhC1r̄ was reduced, alkylated and subjected to size exclusion chromatography. The A and B chains (23) were separated (Figure 3, experiment II). The A chain, which possesses the only consensus sequence-containing EGF module, was hydroxylated to 11%, whereas the B chain did not contain eHya. The position of eHya within the A chain was determined in a third

A



B

PEAK	SEQUENCE	Hya (pmol)	Pep (pmol)	Hya/Pep
	1 20 40			
EGF-S_{2B}	DINECKDPVNINGGCSQICE**N**TPGSYHCSCKNGFVMLSNKKDCKDV	550	4230	0.13 ± 0.02
1	**N**TPGSYHCSCKNGFVMLSNKKDCKDV (72%) GFVMLSNKKDCKDV (28%)	268	1790	0.15 ± 0.01
1, following one cycle of Edman degradation	TPGSYHCSCKNGFVMLSNKKDCKDV FVMLSNKKDCKDV	≤2		≤0.002
2	**N**TPGSYHCSCKNG	118	850	0.14 ± 0.03
3	**N**TPGSYHCSCKNGFVML	56	470	0.12 ± 0.03
4	NING (34%) **N**TPGSYHCSCKNG (26%) GFVMLSN (40%)	29	240	0.12 ± 0.06
5	CKDPVNINGGCSQICE (35%) **N**TPGSYHCSCKNGFV (30%) GFVMLSNKKDCKDV (35%)	17	155	0.11 ± 0.20
6	DINECKDPVNI (38%) CKDPVNI (18%) **N**TPGSYHCSC (18%) FVMLSNK (25%)	≤2	95	≤0.02
7	**N**TPG (15%) (85%) CKDV	≤2	60	≤0.03
8	DINE(CKD) (83%) (17%) NKKD(CKD)	≤2	0	---
9	CKDPVNINGGCSQICE	≤2	0	---

Figure 2. Identification of the site of hydroxylation within EGF-S_{2B}. A. Twenty eight nmoles of partially hydroxylated EGF-S_{2B} (0.13 ± 0.02 mol eHya/mol peptide) were isolated from Sf9 assay mixtures by reverse phase HPLC, derivatized and reisolated by reverse phase HPLC as described in Experimental Procedures. The derivatized peptide was digested with endoproteinase Glu-C and the resulting digest was resolved by reverse phase HPLC (17), in which 92% of the loaded eHya was recovered. Peaks were numbered according to decreasing eHya content. B. The amino acid sequence of the peptides within each peak as well as their eHya content are shown. Peptide content was calculated using amino acid analysis. The relative ratio of peptides within eHya-containing peaks was determined by amino acid and sequence analyses, and is indicated in parentheses. Molar ratios of eHya to peptide were calculated using only the amount of asn 21-containing peptide, which is indicated as PEP. Equal portions (107 pmol eHya) of peak 1 were applied to two sequence discs; one was washed and hydrolyzed (control disc), the other was subjected to one cycle of Edman degradation and then hydrolyzed. In the control disc, 94 pmol of eHya were recovered, with eHya/peptide = 0.10 ± 0.01. Less than 2 pmol of eHya (eHya/peptide ≤ 0.002) were recovered from the other disc. The degree of uncertainty of the eHya/peptide ratio of peak 5 results from the error involved in determining the amount of asn 21-containing peptide in a sample in which the total peptide concentration is low (12% of each peak was analyzed for amino acid composition; the remainder was subjected to eHya and sequence analyses). The small amount of eHya expected in peaks 6 and 7 (10 pmoles each, 3.5% of total eHya loaded) was not detected. The CKD sequence shown in parentheses in peak 8 may belong to one peptide or both. However, based on the expected endoproteinase Glu-C cleavage site and the quantitative recovery of peptide fragments, the majority of the CKD sequence likely follows NKKD. A dashed line indicates no ratio could be calculated due to the absence of peptide containing asn 21. Peak 10 possessed no detectable eHya or sequence.

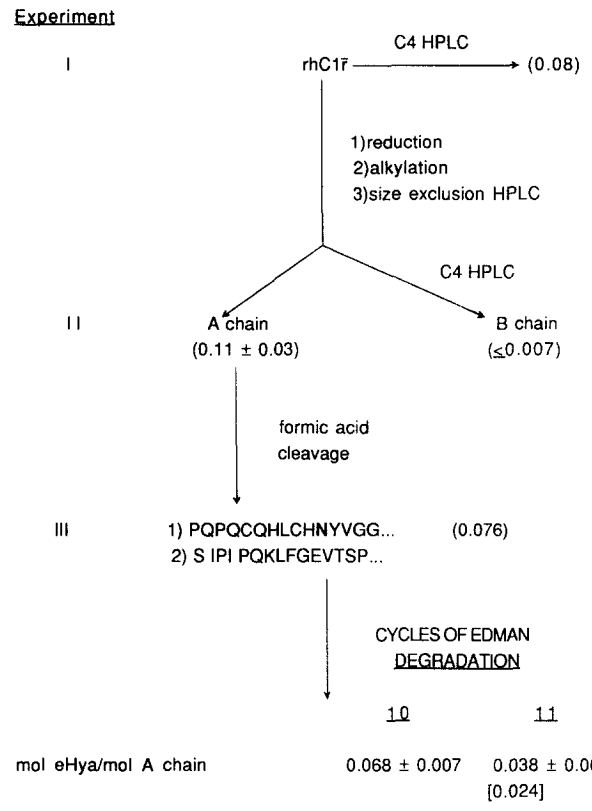


Figure 3. Identification of the site of hydroxylation within recombinant human C1r. See text for description of experments I, II and III. Molar ratios of eHya to protein or peptide are indicated in parentheses (mean ± range is shown when duplicate determinations were made), and were calculated using 73 asp and asn (asx) residues/rhC1r, 42 asx/A chain and 41 asx/A chain following 10 and 11 cycles of Edman degradation, respectively. Asx determinations were obtained from eHya analyses as previously described (17). [0.024] represents the corrected molar ratio of eHya/A chain remaining on the sequencing disc following 11 cycles of Edman degradation, taking into account an Edman reaction efficiency of 96%/cycle determined in the same analysis.

experiment (Figure 3, experiment III). Treatment of the A chain with formic acid generated a new amino terminus 11 residues from the expected position of hydroxylation (asn 150). Edman degradation of equal portions of this cleavage mixture through 10 or 11 cycles, respectively, revealed that while no significant eHya was removed following 10 cycles, approximately 70% of the eHya (corrected for 96% Edman reaction efficiency at each cycle and normalized for peptide content) was removed at the 11th cycle. Although this method allowed for the assignment of approximately 70% of the eHya to the expected position, its inherent limitations preclude us from assigning the position of the remaining eHya, which may be at the expected position or elsewhere.

Since EGF-S_{2B} and rhC1r contain asn residues at the positions of hydroxylation, we determined whether the invertebrate preparations also hydroxylated EGF modules that contained a corresponding asp residue at this site. For this, two peptides, the structures of which are based on the first EGF module of human factor X and contain either asp (EGF-X_{1H}) (18) or asn

(EGF-X_{1H}-asn) (18, 19) at the hydroxylation site were used. In the Sf9 hydroxylase system, EGF-X_{1H} generated 5% and EGF-X_{1H}-asn 20% of the activity observed with EGF-S_{2B} (CO_2 and eHya data not shown). Similarly, EGF-X_{1H} generated 5% and EGF-X_{1H}-asn 40% of the S2 hydroxylase activity observed with EGF-S_{2B}. These data suggest that invertebrate hydroxylases can hydroxylate asp-containing EGF modules but, unlike the mammalian enzyme (19), may have an intrinsic preference for asn-containing EGF modules. An alternative explanation for the relatively low reactivity of the asp-containing substrates is that the structure of the factor X EGF module may not be optimal for asp hydroxylation by the invertebrate hydroxylases. In this regard, human factor VII, which contains a consensus sequence within an EGF module, is not hydroxylated in vivo (24), suggesting that the consensus sequence alone is not sufficient for hydroxylase recognition. It is not known whether the minimum structural requirements for hydroxylation result from specific primary structure determinants, disulfide bond arrangements, or the absence of negative interactions with other regions of the intact protein. Studies with the mammalian hydroxylase have shown that primary structure is an important determinant for k_{cat}/K_m values of substrates with identical disulfide bond patterns (18). Unpublished observations[1] indicated that EGF modules which differ only in their disulfide bond pairing are substrates for the mammalian hydroxylase, suggesting that a unique secondary structure is not absolutely required by the hydroxylase. The disulfide bond arrangement of EGF-S_{2B}, which was hydroxylated at the expected position by the Sf9 hydroxylase, has not been determined. Clearly, further studies are required to determine the minimum structural requirements for substrate recognition by the invertebrate and mammalian enzymes.

Proteins from both vertebrates and invertebrates have been shown to contain homologous EGF modules that are involved in direct protein-protein interactions (1). Some of these modules contain the consensus sequence for hydroxylation. For example, Notch (10), a protein required for cell communication at many stages of *D. melanogaster* development, contains 36 EGF modules, 22 of which possess the consensus sequence. Genes coding for proteins that are homologous to Notch have also been identified in *C. elegans* (11), *S. purpuratus* (16), *Xenopus* (25), rat (26) and human cells (27). Studies with the Notch gene product suggest that it may act as a multifunctional receptor whose EGF modules form a tandem array of discrete ligand-binding units, each of which may potentially interact with several different proteins during development (28, 29). TAN-1, the human homolog of the Notch gene may be important for normal T lymphocyte function; it has been suggested that alterations of this gene may play a role in the pathogenesis of some T cell neoplasms (27). Our present studies have demonstrated that like the EGF modules themselves, the enzymatic activity that can potentially hydroxylate specific asn and asp residues within some of these modules has been conserved from insects to mammals. Therefore, it is possible that posttranslational hydroxylation of certain EGF modules may be important in regulating EGF module-dependent protein-protein interactions and the physiologic signals that ultimately result from these

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interactions. These studies provide the basis for further investigations concerned with identifying hydroxylated invertebrate proteins and determining their physiologic function.

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