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Biochemistry

Hydroxylation of aspartic acid in domains homologous to the epidermal growth factor precursor is catalyzed by a 2-oxoglutarate-dependent dioxygenase

(3-hydroxyaspartic acid/epidermal growth factor-like domains)

JOHAN STENFLO*, ELISABETH HOLME†, SVEN LINDSTEDT†, N. CHANDRAMOULI‡, LINDA H. TSAI HUANG‡, JAMES P. TAM‡, AND R. B. MERRIFIELD‡

*Department of Clinical Chemistry, University of Lund, Malmö General Hospital, S-214 01 Malmö, Sweden; †Department of Clinical Chemistry, Gothenburg University, Sahlgren's Hospital, S-413 45 Gothenburg, Sweden; and ‡The Rockefeller University, 1230 York Avenue, New York, NY 10021

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ABSTRACT **3-Hydroxyaspartic acid and 3-hydroxyasparagine are two rare amino acids that are present in domains homologous to the epidermal growth factor precursor in vitamin K-dependent plasma proteins as well as in proteins that do not require vitamin K for normal biosynthesis. They are formed by posttranslational hydroxylation of aspartic acid and asparagine, respectively. The first epidermal growth factor-like domain in factor IX (residues 45–87) was synthesized with aspartic acid in position 64, replacing 3-hydroxyaspartic acid. It was used as substrate in a hydroxylase assay with rat liver microsomes as the source of enzyme and reaction conditions that satisfy the requirements of 2-oxoglutarate-dependent dioxygenases. The synthetic peptide stimulated the 2-oxoglutarate decarboxylation in contrast to synthetic, modified epidermal growth factor (Met-21 and His-22 deleted and Glu-24 replaced by Asp) and synthetic peptides corresponding to residues 60–71 in human factor IX. This indicates that the hydroxylase is a 2-oxoglutarate-dependent dioxygenase with a selective substrate requirement.**

3-Hydroxyaspartic acid is an uncommon amino acid, first identified in domains that are homologous to the epidermal growth factor (EGF) precursor in the vitamin K-dependent plasma proteins, protein C, factor IX, and factor X (1–5). More recently 3-hydroxyasparagine was found in vitamin K-dependent protein S, which contains one 3-hydroxyaspartic acid residue and three 3-hydroxyasparagine residues (6). 3-Hydroxyaspartic acid and 3-hydroxyasparagine, which are both in the erythro form in proteins, are formed by posttranslational hydroxylation of aspartic acid and asparagine, respectively (1, 6). The modified amino acids have now also been found in EGF-homologous regions in proteins that do not require vitamin K for normal biosynthesis—i.e., the complement proteins C1r and C1s, the low density lipoprotein receptor, thrombomodulin, thrombospondin, and uromodulin (6–8). The function of the hydroxylated amino acids has not yet been definitely established. However, several lines of evidence suggest that 3-hydroxyaspartic acid is required for normal Ca^{2+} binding and biological activity in protein C and the related vitamin K-dependent plasma proteins (2, 3, 9–13).

In mammals 2-oxoglutarate-dependent dioxygenases catalyze hydroxylation of protein-bound proline (14, 15), lysine (16), and N^{ϵ}-trimethyllysine (16) as well as of free γ-butyrobetaine (17). Trimethyllysine hydroxylase (EC 1.14.11.8) is a mitochondrial enzyme (18) and γ-butyrobetaine hydroxylase (EC 1.14.11.1) is a cytosolic enzyme (19). Prolyl 4-hydroxylase (EC 1.14.11.2), prolyl 3-hydroxylase (EC 1.14.11.7), and lysyl 5-hydroxylase (EC 1.14.11.4) are present in the microsomes (20, 21). The natural substrate for these enzymes is procollagen. Prolyl 4-hydroxylase and lysyl 5-hydroxylase also hydroxylate oligopeptides with a minimum chain length of six residues and the sequence Xaa-Pro/Lys-Gly (21). It has been established (22) that bacterial γ-butyrobetaine hydroxylase utilizes 2-oxoglutarate as a reducing agent for molecular oxygen with the formation of 1 mol of succinate and 1 mol of CO_2 per mol of hydroxylated product. This made it possible to measure the activity of this group of enzymes by following the substrate-dependent CO_2 formation from 2-oxoglutarate.

It appeared possible that the enzyme that hydroxylates certain aspartic acid and asparagine residues in EGF homologous regions is also a 2-oxoglutarate-dependent enzyme. To design a substrate for the hydroxylase, we have compared the amino acid sequence of EGF homologous regions that contain 3-hydroxyaspartic acid or 3-hydroxyasparagine, with the sequence of such domains that do not have the modified amino acids (6). The comparison enabled us to identify a consensus sequence. Cys-Xaa-Asp/Asn-Xaa-Xaa-Xaa-Xaa-Phe/Tyr-Xaa-Cys-Xaa-Cys, corresponding to residues 62–73 in human factor IX, in all of the proteins in which aspartyl and asparaginyl residues are hydroxylated. Peptides containing residues 60–71 were synthesized as well as two high molecular weight substrates—i.e., the first EGF-like domain of human factor IX and murine EGF with two residues deleted (Met-21 and His-22) and one glutamic acid residue (Glu-24) replaced by aspartic acid (Fig. 1).

We now describe experiments indicating that the enzyme which hydroxylates one aspartic acid residue in the first EGF-like unit of factor IX is a 2-oxoglutarate-dependent dioxygenase.

MATERIALS AND METHODS

The peptides (*i*) acetyl-Lys-Asp-Asp-Ile-Asn-Ser-Tyr-Glu-Cys-NH_2 (corresponding to residues 63–71 in factor IX), (*ii*) H-Gly-Ser-Cys-Lys-Asp-Asp-Ile-Asn-Ser-Tyr-NH_2 (corresponding to residues 60–69 in factor IX), and (*iii*) H-Gly-Ser-Ala-Lys-Asp-Asp-Ile-Asn-Ser-Tyr-Glu-Cys-NH_2 (corresponding to residues 60–71 in factor IX but with an alanine in position 62 instead of cysteine) were synthesized by Ferring Pharmaceuticals (Malmö, Sweden) using solid-phase methodology on an Applied Biosystems peptide synthesizer. The peptides were purified to homogeneity by HPLC. 2-Oxo[1-^{14}C]glutarate was from New England Nuclear.

Synthesis of the First EGF-Like Domain (Residues 45–87) from Factor IX. The factor IX EGF-like domain was synthe-

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Abbreviation: EGF, epidermal growth factor.

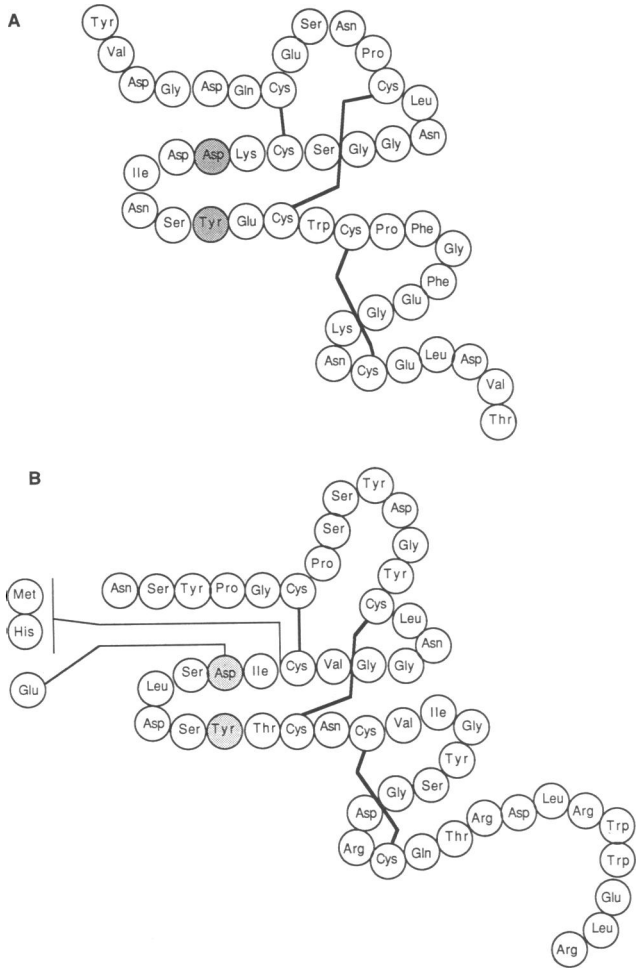

FIG. 1. Schematic representation of the secondary structure (based on ref. 13) of the two synthetic EGF-like domains used as substrate in the hydroxylase reaction. (*A*) The first EGF-like domain of human factor IX (residues 45–87). (*B*) The modified murine EGF. The methionine and histidine residues that were deleted and the glutamic acid residue that was replaced by aspartic acid in the modified molecule are shown to the left. Disulfide bonds are marked with solid lines. Asp-64, which is hydroxylated in factor IX, and Tyr-69, which is thought to be adjacent in the β-sheet (12), are stippled as well as the corresponding residues in the modified murine EGF molecule.

sized manually by the stepwise solid-phase method (23). The synthesis started on N^{α}-Boc-Thr(Bzl)-OCH_2-Pam resin (24) at 0.76 mmol/g of resin (Applied Biosystems), where Boc is *p*-bromobenzyloxycarbonyl, Bzl is benzyl, and Pam is phenylacetamidomethyl. The synthesis was performed with the stepwise addition of Boc amino acids using standard coupling and deprotection techniques (23, 25, 26). The completed peptide was deblocked by treatment with trifluoroacetic acid in CH_2Cl_2 [1:1 (vol/vol)] to remove the NH_2-terminal Boc protecting groups. To remove the peptide from the resin the low/high HF method of cleavage was used (27). After removal of the HF the residue was washed with cold ethyl ether/mercaptoethanol [98:2 (vol/vol)] to remove organic contaminants. The peptide was then extracted with 8 M urea/0.2 M dithiothreitol/0.1 M Tris·HCl, pH 8.4.

Prior to refolding, the peptide solution was dialyzed (Spectra/por 6 tubing; molecular weight cut off, 1000) against deaerated and N_2-purged 8 M, 5 M, 3 M, and 1.5 M urea, all in 0.1 M Tris·HCl (pH 8.45). No precipitation was observed during the dialysis. Oxidation and refolding of the peptide was based on the approach devised by Heath and Merrifield (25). Initially 0.1 M Tris·HCl (pH 8.45) was added to the peptide solution to a final urea concentration of 0.3 M and reduced and oxidized glutathione were added, both to a final concentration of 1 mM. The solution was stirred slowly at 4°C. No precipitate formed during the oxidation process. The reoxidized peptide was purified to homogeneity by preparative HPLC using a Vydac C_{18} column and a linear gradient of 5–60% (vol/vol) acetonitrile/0.05% trifluoroacetic acid.

The amino acid composition of an acid hydrolysate of the synthetic EGF-like domain from factor IX was in agreement with the theoretical value within experimental error. When the synthetic fragment was subjected to ^{252}Cf fission fragment ionization mass spectrometry, a molecular ion $(M + H)^+$ of 4750.6 was obtained, which corresponds closely to the calculated mean value m/z 4749.9. The disulfide bond pairing was determined after digestion of the fragment with thermolysin (28). The peptides were separated by HPLC on an analytical Vydac C_{18} column with a 5–60% (vol/vol) acetonitrile/0.05% trifluoroacetic acid gradient. Two fragments were characterized. One corresponded to the peptide Asp-Gly-Asp-Gln-Cys-Glu linked to Gly-Ser-Cys (residues 47–52 and 60–62 in human factor IX) whereas the other one corresponded to the peptide Cys-Pro linked to Glu-Gly-Lys-Asn-Cys-Glu (residues 73–74 and 78–83 in factor IX).

Synthesis of the Modified Murine EGF. The synthesis of the modified EGF (Fig. 1) was carried out on an Applied Biosystems peptide synthesizer (model 430A) by the stepwise addition of Boc amino acids by using standard coupling and deprotection techniques (23, 25). The completed polypeptide resin was deprotected with trifluoroacetic acid/CH_2Cl_2 [1:1 (vol/vol)] to remove the NH_2-terminal Boc protecting group. The resin was then treated with low/high HF to cleave the peptide (27). The peptide was extracted with 0.1 M Tris·HCl containing 8 M urea and 0.1 M dithiothreitol, pH 8.6. Oxidation and refolding of the peptide was carried out as before (25). The solution was transferred to Spectra/por 6 dialysis tubing and dialyzed for 48 hr against 8 M urea containing 0.1 M Tris·HCl and 20 mM dithiothreitol (pH 8.6). The dialysis was then carried out successively against buffers containing 4 M urea and 2 M urea with 0.1 M Tris·HCl and 20 mM 2-mercaptoethanol (pH 8.6). The last dialysis was carried out against the Tris buffer containing 2 M urea but without 2-mercaptoethanol. Finally, this solution was diluted to a peptide concentration of 0.5 mg/ml with 2 M urea and the disulfide bonds were allowed to form by oxidation in air.

The peptide was purified to homogeneity by reversed-phase HPLC using a Vydac C_{18} column, with a linear gradient from 18 to 66% acetonitrile in 0.45% trifluoroacetic acid in 45 min. The peak was at 20. 7 min (30.8% CH_3CN). The peptide had the correct molecular weight (5757.3) based on mass spectral determination and it had the correct amino acid composition. The disulfide bond pairing was determined on a thermolysin digest of the synthetic peptide as described by Savage *et al.* (28). The modified EGF showed binding to the EGF receptor, induced tyrosine kinase activity, and was active in the thymidine incorporation assay, indicating thereby that the molecule has retained an overall structure similar to the murine EGF (25).

Enzyme Preparation. Fresh rat liver was cut into pieces and homogenized in a Potter–Evehjelm homogenizer in 0.025 M potassium phosphate buffer (pH 7.2) containing 0.25 M sucrose and chymostatin (10 μg/ml), pepstatin (10 μg/ml), and leupeptin (10 μg/ml) (3 g of tissue in 6 ml of buffer). The homogenate was centrifuged for 10 min at 12,000 × *g*. The supernatant (3 ml) was then centrifuged for 1 hr at 100,000 × *g* and the pellet was suspended in 3 ml of 0.05 M potassium phosphate (pH 7.2) containing 0.1% Triton X-100 and each of the three protease inhibitors above at 10 μg/ml. The suspension was frozen in liquid nitrogen and stored at −70°C.

Enzyme Assay. The enzyme activity was measured as the peptide-dependent evolution of $^{14}CO_2$ from 2-oxo[1-^{14}C]-glutarate. The incubations were made in 0.03 M potassium phosphate (pH 7.2) containing 0.06% Triton X-100, 0.05–0.1 mM peptide, 0.25 mM 2-oxo[1-^{14}C]glutarate (148 Bq/nmol), catalase (2 mg/ml), 5 mM sodium ascorbate, 0.5 mM ferrous sulfate, and chymostatin, pepstatin, and leupeptin (each at 10 μg/ml). The enzyme (microsomal suspension) concentration was 5.4–16.2 mg of protein per ml. The incubations were carried out at 37°C for 30–60 min, in a total volume of 50 μl, in 1.5-ml conical Eppendorf tubes. The reactions were stopped by the addition of 50 μl of 2 mM sodium acetate (pH 4.7) containing 30 mM diethyldithiocarbamate. The evolved $^{14}CO_2$ was collected in Hyamine applied on the stopper of the Eppendorf tube. The stopper was then cut off and placed in a scintillation vial containing a solution of 2,5-diphenyloxazolone (10 g), 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene (0.3 g), toluene (1000 ml), and methoxyethanol (600 ml).

Immunochemical Procedure. A rabbit antiserum against human factor IX (Dako, Santa Barbara, CA) immunoprecipitated the NH_2-terminal ^{125}I-labeled EGF-like domain in a double-antibody precipitation procedure at a dilution of 1:1000. The anti-factor IX antibodies were affinity-purified on a column with immobilized human factor IX and then coupled to CNBr-activated Sepharose 4B, at ≈5 mg of antibody per ml of gel, according to the instructions of the manufacturer. The gel was packed in a column (1.5 × 5 cm) and equilibrated with 50 mM Tris·HCl/0.1 M NaCl, pH 8.0, containing 1% lubrol. The incubation mixture (pH 4.7) was diluted with 1 ml of 0.3 M Tris·HCl (pH 8.0) containing 1% lubrol and centrifuged in an Eppendorf microfuge for 1 min, and the supernatant was then applied to the column that was developed at a flow rate of 40 ml/hr. The column was washed with several column volumes of the equilibration buffer and then material was eluted with 50 mM sodium acetate (pH 4.0) containing 0.5 M NaCl and finally with 0.1 M glycine·NaOH containing 0.5 M NaCl (pH 2.2). The material eluted with the glycine buffer was dialyzed against 10% (vol/vol) acetic acid and evaporated to dryness, and 3-hydroxyaspartic acid was measured as described (29).

RESULTS

Human factor IX has 3-hydroxyaspartic acid in position 64 in its first EGF-like domain. We, therefore, used three synthetic peptides, corresponding to residues 63–71, 60–69, and 60–71 in factor IX as substrate for the hydroxylase. None of them stimulated decarboxylation of 2-oxo[1-^{14}C]glutarate. Thus the enzyme may require a substrate with intact disulfide bonds and a native conformation. Therefore, two larger disulfide-containing peptides were synthesized and examined to determine whether they functioned as substrates for the hydroxylase. One of these corresponded to the first EGF-like domain in factor IX and the other one to murine EGF (Fig. 1). The peptide that corresponds to the first EGF-like domain from factor IX (residues 45–87) is a substrate for the enzyme. A 2-oxoglutarate decarboxylase activity dependent on the presence of this substrate was found in the microsomal fraction in several experiments (Table 1). The time progress curve for the reaction with the EGF-like domain from human factor IX showed that the CO_2 evolution was almost twice that observed in incubation mixtures without peptide and increased with time (Fig. 2).

Table 1. Subcellular distribution of 2-oxoglutarate decarboxylase activity in the presence and absence of the synthetic first EGF-like domain of human factor IX

Subcellular fraction	Peptide-independent CO_2, nmol	Peptide-dependent CO_2, nmol
Homogenate	3.0	—
Pellet (12,000 × *g*)	4.6	—
Supernatant (12,000 × *g*)	0.64	0.28
Pellet (100,000 × *g*)	0.42	0.25

A 30% (wt/vol) rat liver homogenate was fractionated by centrifugation. The pellets were diluted in buffer to the original volume. The concentration of peptide was 0.1 mM. The incubation time was 30 min.

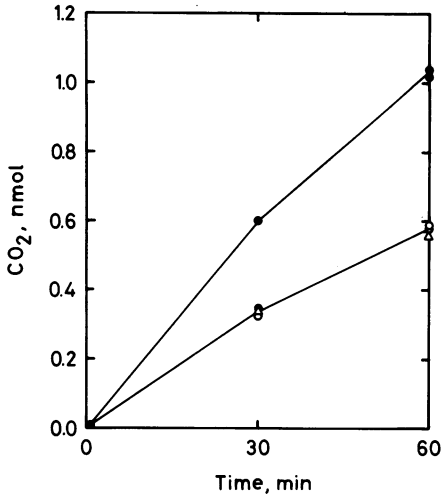

FIG. 2. Time progress curves of 2-oxo[1-^{14}C]glutarate decarboxylation with synthetic EGF-like domains as substrate. ●, EGF-like domain of human factor IX; △, modified murine EGF; ○, control incubations without substrate. The substrate concentration was 0.1 mM and rat liver microsomes (0.39 mg of protein) were used as a source of enzyme. The volume of the incubation mixture was 50 μl.

The dependency of the CO_2 evolution from 2-oxoglutarate on the enzyme concentration is shown in Fig. 3. In incubation mixtures containing the EGF-like domain from factor IX (0.05 mM), there was a significant protein-dependent increase in CO_2 evolution, when compared to those lacking substrate. In contrast there was no difference in CO_2 evolution between incubation mixtures without peptide and those that contained the modified murine EGF (0.10 mM) (Figs. 2

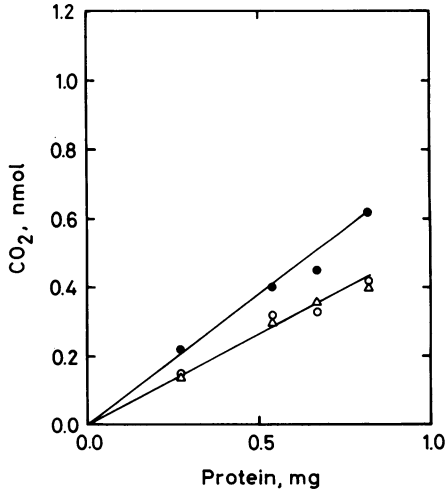

FIG. 3. 2-Oxo[1-^{14}C]glutarate decarboxylation with various amounts of enzyme in the presence of synthetic EGF-like domains. ●, EGF-like domain from human factor IX (0.05 mM); △, modified murine EGF (0.1 mM); ○, control incubations without substrate. Rat liver microsomes were used as substrate. The abscissa gives mg of protein per 50 μl of incubation mixture. The incubation time was 30 min.

and 3). The CO_2 formed with the first EGF-like domain from factor IX as substrate corresponded to 3–8% hydroxylation of the substrate if one assumes 1:1 stoichiometry between the putative hydroxylation and the 2-oxoglutarate decarboxylation.

Attempts were made to isolate the reaction product (i.e., the presumably hydroxylated factor IX EGF-like domain) from the incubation mixtures by using the affinity-purified antibodies against factor IX. The antibodies, which precipitated both ^{125}I-labeled factor IX and the factor IX EGF-like domain in a double-antibody precipitation assay, had been immobilized on Sepharose 4B beads. The antibodies bound 3-hydroxyaspartic acid-containing material both from incubation mixtures that contained substrate and from control incubation mixtures, presumably due to a crossreaction of the antibodies with rat factor IX and perhaps other rat vitamin K-dependent proteins. The isolation and characterization of the reaction products awaits the synthesis of the factor IX substrate in larger amount and a partial purification of the enzyme.

DISCUSSION

We have demonstrated a significant peptide-dependent decarboxylation of 2-oxoglutarate with a synthetic substrate identical with the first EGF-like domain of factor IX, except that 3-hydroxyaspartic acid in position 64 had been replaced by aspartic acid. Progress of the reaction appeared to be linear both with respect to time and enzyme concentration.

Attempts to isolate a putative hydroxylated product from the crude incubation mixtures were unsuccessful due to the small amount of substrate available. Possible causes for the 2-oxoglutarate decarboxylation other than an aspartyl hydroxylase-catalyzed reaction must, therefore, be considered. The only known peptide-dependent 2-oxoglutarate decarboxylase activity that occurs in rat liver microsomes in amounts comparable to the activity found, is prolyl hydroxylase (30). However, prolyl hydroxylation is highly unlikely since the first EGF-like domain of factor IX does not have any hydroxyproline residue and it lacks the amino acid sequence known to be required for prolyl hydroxylation—i.e., Xaa-Pro-Gly (21). Furthermore, the modified EGF, which has the required amino acid sequence (residues 3–5), did not stimulate the 2-oxoglutarate decarboxylation. Uncoupling (i.e., decarboxylation of 2-oxoglutarate without concomitant hydroxylation) has been demonstrated in reactions catalyzed by proline (31), γ-butyrobetaine (32, 33), and thymine (34, 35) hydroxylases in the presence of substrate analogues. With the natural substrates, however, there is a close coupling between proline (14) and lysine hydroxylation (36) and 2-oxoglutarate decarboxylation. We, therefore, regard it as highly unlikely that the 2-oxoglutarate decarboxylation in incubation mixtures containing the EGF-like domain from factor IX is caused by an uncoupled dioxygenase reaction.

The three-dimensional structure of human EGF was determined by ^{1}H NMR spectroscopy by Cooke *et al.* (13). They also pointed out that the Tyr/Phe residue that is conserved in the consensus sequence is positioned adjacent to the 3-hydroxyaspartic acid residue on the same face of a β-sheet. Work of Heath and Merrifield (25) indicates that properly folded EGF is essential for biological activity and receptor binding. The failure of the nona-, deca-, and dodecapeptides to stimulate 2-oxoglutarate decarboxylation suggests that a properly folded substrate also is required for aspartic acid hydroxylation. However, the failure of the modified EGF to stimulate 2-oxoglutarate decarboxylation indicates that the consensus sequence in a properly folded β-sheet is not sufficient to satisfy the stringent substrate requirements for aspartic acid hydroxylation.

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