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Initiation of Chondroitin Sulfate Biosynthesis: A Kinetic Analysis of UDP-D-Xylose:Core Protein β-D-Xylosyltransferase†

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ABSTRACT: The nature of the primary signals important for the addition of xylose to serines on the core protein of the cartilage chondroitin sulfate proteoglycan has been investigated. The importance of consensus sequence elements (Acidic-Acidic-Xxx-Ser-Gly-Xxx-Gly) in the natural acceptor was shown by the significant decrease in acceptor capability of peptide fragments derived by digestion of deglycosylated core protein with *Staphylococcus aureus* V8 protease, which cleaves within the consensus sequence, compared to the similar reactivity of trypsin-derived peptide fragments, in which consensus sequences remain intact. A comparison of the acceptor efficiencies (V_{max}/K_m) of synthetic peptides containing the proposed xylosylation consensus sequence and the natural acceptor (deglycosylated core protein) was then made by use of the in vitro xylosyltransferase assay. The two types of substrates were found to have nearly equivalent acceptor efficiencies and to be competitive inhibitors of each other's acceptor capability, with $K_m = K_i^{apparent}$. These results suggest that the artificial peptides containing the consensus sequence are analogues of individual substitution sites on the core protein and allowed the kinetic mechanism of the xylosyltransferase reaction to be investigated, with one of the artificial peptides as a model substrate. The most probable kinetic mechanism for the xylosyltransferase reaction was found to be an ordered single displacement with UDP-xylose as the leading substrate and the xylosylated peptide as the first product released. This represents the first reported formal kinetic mechanism for this glycosyltransferase and the only one reported for a nucleotide sugar:protein transferase.

Chondroitin sulfate proteoglycan (CSPG),[1] a major component of cartilage extracellular matrix, has a protein core of apparent molecular mass 340–370 kDa and is substituted with complex carbohydrates up to 10 times its protein mass (Upholt et al., 1979; Campbell & Schwartz, 1988). The linkage region of the characteristic chondroitin sulfate chain of CSPG consists of a serine-linked xylose–galactose–galactose–glucuronic acid tetrasaccharide followed by the repeating disaccharide, *N*-

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[1] Abbreviations: CSPG, chondroitin sulfate proteoglycan; HPLC, high-pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid; PGHF, chondroitin sulfate proteoglycan deglycosylated with hydrogen fluoride; CS, chondroitin sulfate; CS,B, peptide B from chondroitin sulfate domain of core protein; CS,B-HF, CS,B deglycosylated with hydrogen fluoride; SDS, sodium dodecyl sulfate; GAG, glycosaminoglycan.

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acetylgalactosamine–glucuronic acid (Rodén & Smith, 1966). The transfer of D-xylose from UDP-xylose to certain serine hydroxyl groups of the core protein is catalyzed by the chain-initiating enzyme, xylosyltransferase. Several lines of evidence suggest a regulatory role for xylosyltransferase in CSPG biosynthesis. (1) The extensive posttranslational modification of the core protein that is necessary for complete transport and secretion of CSPG is initiated by xylosyltransferase (Schwartz, 1979). (2) Core protein synthesis and xylosyltransferase activity appear to be coordinated (Schwartz, 1976). (3) The specific interaction of xylosyltransferase with galactosyltransferase I (Schwartz & Rodén, 1974; Schwartz & Dorfman, 1975), the next enzyme in the pathway, may be physiologically important in transporting and directing the core protein to further processing (Campbell & Schwartz, 1988). Therefore, a complete investigation of the nature and mechanism of the xylosyltransferase reaction is important to our understanding of the regulation of CSPG biosynthesis.

Although xylosyltransferase has been purified to homogeneity (Schwartz & Rodén, 1974; Schwartz & Dorfman, 1975), detailed studies of the reaction have been hindered in the past by a lack of information about the core protein substrate. This large protein contains approximately 300 serine residues of which about 80–100 are substituted with chondroitin sulfate chains. It has only recently become clear that these 80–100 chondroitin sulfate substituted serines are tightly clustered within a specific domain of the core protein and may be spaced as closely as three to five amino acid residues apart (Krueger et al., 1990a,b). Proximity of acceptor sites may be an important feature of acceptor specificity that was previously unrecognized and, therefore, never addressed directly.

Furthermore, the primary features of the core protein that mark the 80–100 specific serines for xylose substitution in the large proteoglycans have not yet been identified. An amino acid consensus sequence for formation of the xylose–serine linkage consisting of Glu/Asp-Glu/Asp-Xxx-Ser-Gly-Xxx-Gly has been proposed (Bourdon et al., 1987) for certain of the small proteoglycans. This sequence was arrived at by comparing the amino acid sequence around known or presumed substituted serines from three proteoglycan core proteins and then testing synthetic peptides containing this sequence or modifications of it as acceptors in the xylosyltransferase assay. However, until very recently, no evidence has been presented to support its existence in the complex multiply substituted proteoglycans. As well, it is not known whether the proposed xylosylation consensus sequence is both necessary and sufficient for glycosylation to occur.

The most definitive information about the primary protein structure around xylose-substituted serines has come from direct protein sequencing through multiple substitution sites, before and after deglycosylation, of peptides derived from the cartilage CSPG core protein. Comparison of this direct protein sequence with the proposed consensus sequence shows that while not every substituted serine is part of a complete consensus sequence, nearly every substituted serine is surrounded by at least one of the putative essential features (Krueger et al., 1990a,b). This work provides the first direct evidence that the consensus sequence features previously elucidated in small proteoglycans are important for xylose addition in the multiply substituted proteoglycans as well. Knowing that the majority of the substitution sites in this multiply substituted core protein use one or the other feature of the consensus sequence (preceding acidic residues or trailing Xxx-Gly sequence), several important aspects of this key reaction were able to be addressed by use of artificial peptides containing the consensus sequence. The first is whether the synthetic peptides and each site in the natural core protein are kinetically comparable, and the second is whether there is an influence of one acceptor site on another or whether they are acted upon independently during the xylosylation process. Furthermore, the artificially defined peptides were invaluable for investigating the kinetic mechanism of xylosyltransferase, not previously possible because of the poorly defined nature of the core protein substrate and the inability to control protein substrate concentration precisely.

Experimental Procedures

Materials

UDP-[^{14}C]xylose (258.7 mCi/mmol) was purchased from New England Nuclear. UDP-xylose, UDP, and V8 protease were purchased from Sigma. Tryspin was obtained from ICN Immunobiologicals. Sephadex G-100 was obtained from Pharmacia. Ion-exchange resin AG50W-X2 (H^+ form, 200–400 mesh) was purchased from Bio-Rad.

Methods

Peptide Synthesis. Some of the peptides are analogous to those originally furnished by Dr. E. Ruoslahti (La Jolla Cancer Research Foundation) and were synthesized as previously described (Bourdon et al., 1987). Additional quantities of peptides were synthesized by use of a Du Pont RaMPS multiple peptide synthesis system according to the instruction manual. Peptides were purified by reverse-phase HPLC on an Alltech Macrosphere 300, C4, 7-micron column with 0.1% trifluoroacetic acid. Amino acid analyses of purified peptides were performed to verify the correct composition. Cysteine residues remained in the protected (tBu) form.

Core Protein Deglycosylation. Deglycosylation of rat chondrosarcoma proteoglycan was carried out according to the procedure of Olson et al. (1985). Isolation and deglycosylation of chondroitin sulfate peptide B (CS-B) from chick cartilage was performed as described by Krueger et al. (1990a).

Enzyme Assay. All enzyme assays were carried out with use of partially purified xylosyltransferase as described previously (Bourdon et al., 1987). Reaction mixtures contained the following in a total volume of 100 μL: 48 mM 4-morpholineethanesulfonic acid (MES) buffer (pH 6.5), 48 mM KCl, 8.4 mM $MgCl_2$, 11.4 mM $MnCl_2$, 7.5 mM KF, 1.2 nmol of UDP-[^{14}C]xylose, Sephacryl S-200 purified enzyme (≈40 μg of protein), and varying amounts of peptide, unlabeled UDP-xylose, and UDP or PGHF. After incubation at 37 °C for 1 h, reaction products were adsorbed on a 2-mL Pasteur pipette column of AG50W-X2 resin, washed with 3–4 bed volumes of 0.01 N HCl, and eluted with 2 M NH_4OH. Incorporated radioactivity was then measured by liquid scintillation counting. Reactions carried out in the presence of PGHF were first subjected to column chromatography on a 20 × 1 cm column of Sephadex G-100 and eluted with 50 mM sodium acetate, pH 4.5, with 150 mM NaCl. PGHF eluted with the void volume, and the peptides eluted near the total volume. Fractions encompassing the total volume were pooled and then adsorbed to and eluted from a 2-mL Pasteur pipette column of AG50W-X2 and counted as described above. The void volume fractions were pooled separately and counted as well. Incorporated radioactivity was shown to be linear with time through at least 1 h of incubation and with enzyme protein concentration over a broad range including the concentration used here (40 μg/100 μL).

Protease Digestion. PGHF from rat chondrosarcoma (20 μg) was digested with 0.25 mg/mL of trypsin in 0.1 M sodium acetate/Tris buffer, pH 7.3, for 4 h at 37 °C. Phenyl-

methanesulfonyl fluoride (1 mM) was added to inactive the trypsin, and half of the resulting mixture was analyzed by SDS–polyacrylamide gel electrophoresis. The remaining half was mixed with xylosyltransferase, and [^{14}C]xylose incorporation was determined by trichloroacetic acid precipitation of peptides as described (Olson et al., 1985). Appropriate controls were included that demonstrated complete inactivation of trypsin and no inhibition of xylosyltransferase activity under these conditions. Digestion with *Staphylococcus aureus* V8 protease was carried out essentially as described by Houmard and Drapeau (1972). PGHF (50 μg) or CS-B (65 μg) was incubated at 37 °C for 22 h with 318 μg of insoluble V8 protease in 50 mM NH_4CO_3, pH 7.8. After incubation the insoluble protease was removed by centrifugation. The supernatant was lyophilized, reconstituted in 50 mM MES (pH 6.5)/50 mM KCl/3 mM $MnCl_2$/12 mM $MgCl_2$ buffer, and assayed for xylosyltransferase acceptor activity. Controls omitting trypsin or V8 protease were included to assess the acceptor activity prior to digestion. The efficacy of digestion was monitored by SDS–polyacrylamide gel electrophoresis.

Kinetic Analysis. All kinetic data were analyzed by use of the BASIC program ENZKIN on a DEC-20 computer or MacENZKIN version 1.1 on a Macintosh Plus. This program uses an iterative least-squares method to fit the initial velocity data to the best rectangular hyperbola. All data are presented here in double-reciprocal form with lines derived from the computer-fitted best rectangular hyperbola. Inhibitor studies were analyzed similarly. The type of inhibition was determined by assessing whether a statistically significant change in the parameters visualized here as slope and/or intercept values of double-reciprocal plots was observed over a range of inhibitor concentrations. The ratio V_{max}/K_m is a function of acceptor activity, with increased V_{max}/K_m values reflecting higher acceptor activity because of either a high V_{max} or a low K_m. This ratio is used to define the efficiency of an acceptor. Computer modeling was carried out by using the apparent kinetic constants, derived from secondary plots of the best-fit kinetic parameters from the primary plots, with the appropriate rate equation to generate an overall velocity pattern for comparison with the bisubstrate variation data. By these means, the quality of fit of a proposed formal mechanism to the results of a complete bisubstrate variation experiment could be judged. Statistical significance was determined by a simple *t* test program.

Results

Acceptor Activity of HF-Deglycosylated CSPG (PGHF). Although artificial peptides containing the CS consensus sequence have been shown to accept xylose in an in vitro assay, it was necessary to show that the enzyme–substrate interactions of the artificial peptides[2] were kinetically the same as those of the natural xylose acceptor (deglycosylated core protein). A comparison of the xylosyltransferase acceptor activity of HF-deglycosylated chondroitin sulfate proteoglycan (PGHF) with that of the artificial peptides containing the consensus sequence is shown in Table I. PGHF containing approximately 80–100 potential glycosylation sites was found to accept very efficiently in the assay, exhibiting a V_{max}/K_m ratio of 118, which corresponds to an efficiency of approximately 1.2–1.5 per glycosylation site. This acceptor efficiency per site is near the range of the observed V_{max}/K_m values for the peptides.

To determine whether these acceptors act at the same enzymatic site, and, therefore competitively inhibit each other's acceptor activity in a well-defined manner ($K_m = K_i^{apparent}$), cross-inhibition studies were carried out. PGHF and peptides containing the consensus sequence were coincubated in the xylosyltransferase assay to test for their ability to inhibit each other's acceptor activity. After the reaction products were separated by gel filtration, the incorporation of [^{14}C]xylose into each product was quantified as described in the Experimental Procedures section. As shown in Figure 1, in both cases PGHF and the peptides significantly inhibited each other's acceptor activity. The double-reciprocal plots clearly exhibit a pattern (slope effect without intercept effect) indicative of competitive inhibition. Analysis of the data by the ENZKIN program confirmed that the K_m values changed significantly while the V_{max} values remained essentially unaltered (data not shown). The kinetic constants for the productive (K_m) and inhibitory ($K_i^{apparent}$) interactions of each acceptor have been calculated (Figure 1). In both cases no statistically significant difference between the K_m and $K_i^{apparent}$ values was observed, suggesting that the interactions responsible for inhibition were indistinguishable from those leading to product. Hence, the observed competitive inhibition and equivalence of K_m and $K_i^{apparent}$ values in these experiments indicates that both PGHF and the synthetic peptides react at the same enzymatic site.

Table I: Comparison of the Xylosyltransferase Acceptor Activities of HF-Deglycosylated CSPG and Consensus Sequence Peptides

acceptor	K_m (μM)	V_{max} (μM/h)	V_{max}/K_m
peptide 1	120 ± 11	76 ± 11[a]	0.63 ± 0.11
peptide 2	62 ± 5.0	38 ± 0.95	0.61 ± 0.052
peptide 7	790 ± 160	31 ± 3.3	0.039 ± 0.009
PGHF	0.11 ± 0.034	13 ± 1.0	118 ± 38 1.2–1.5[b]

[a] The V_{max} value for peptide 1 is significantly different from the others in this table at >95% confidence level. [b] The acceptor efficiency (V_{max}/K_m) per glycosylation site in the core protein of CSPG can be estimated on the basis of the fact that there are about 80–100 glycosaminoglycan chains, i.e., substitution sites, per core protein (Rodén et al., 1985).

Although the results of the quantitative analysis presented in Table I may also be interpreted to suggest that each acceptor site in a multisite substrate like PGHF acts independently (or is equal in acceptor efficiency), the following experiments offer more definitive support of this interpretation. If the core protein consists of a series of individual acceptor sites surrounded by features of the consensus sequence, Acidic-Acidic-Xxx-Ser-Gly-Xxx-Gly, then disruption of the core protein between sites should yield a mixture of peptides with an acceptor capability the same as that of the intact core protein, while disruption within the consensus sequence should decrease the acceptor capability of the resulting peptides. PGHF and CS-B (a purified CSPG peptide shown to contain only CS chains) after HF treatment (CS-B,HF) were digested with trypsin and *S. aureus* V8 protease, and the acceptor activity of the resulting peptide fragments was determined. Limit digestion of intact PGHF was achieved as determined by a time course of digestion as well as by SDS–polyacrylamide gel electrophoresis in which only fragments of $M_r < 14000$ were seen (data not shown). Treatment with trypsin, which on the basis of sequence analysis should cleave between xylose-accepting sites but not within the consensus sequence, produced a mixture of peptides with 91% of the acceptor activity of that of the same amount of intact PGHF. However, treatment with *S. aureus* V8 protease, which cleaves on the carboxy side of acidic amino acids (Drapeau et al., 1972) and therefore would disrupt the consensus sequence, resulted in a loss of acceptor activity in the fragments as compared to the intact proteins: PGHF fragments, 55% of control; CS-B,HF

[2] Peptide 1 = FMLEDEASGIGP; peptide 2 = CDEASGIGPEVPDDRD; peptide 7 = SDDYSGSGSG.

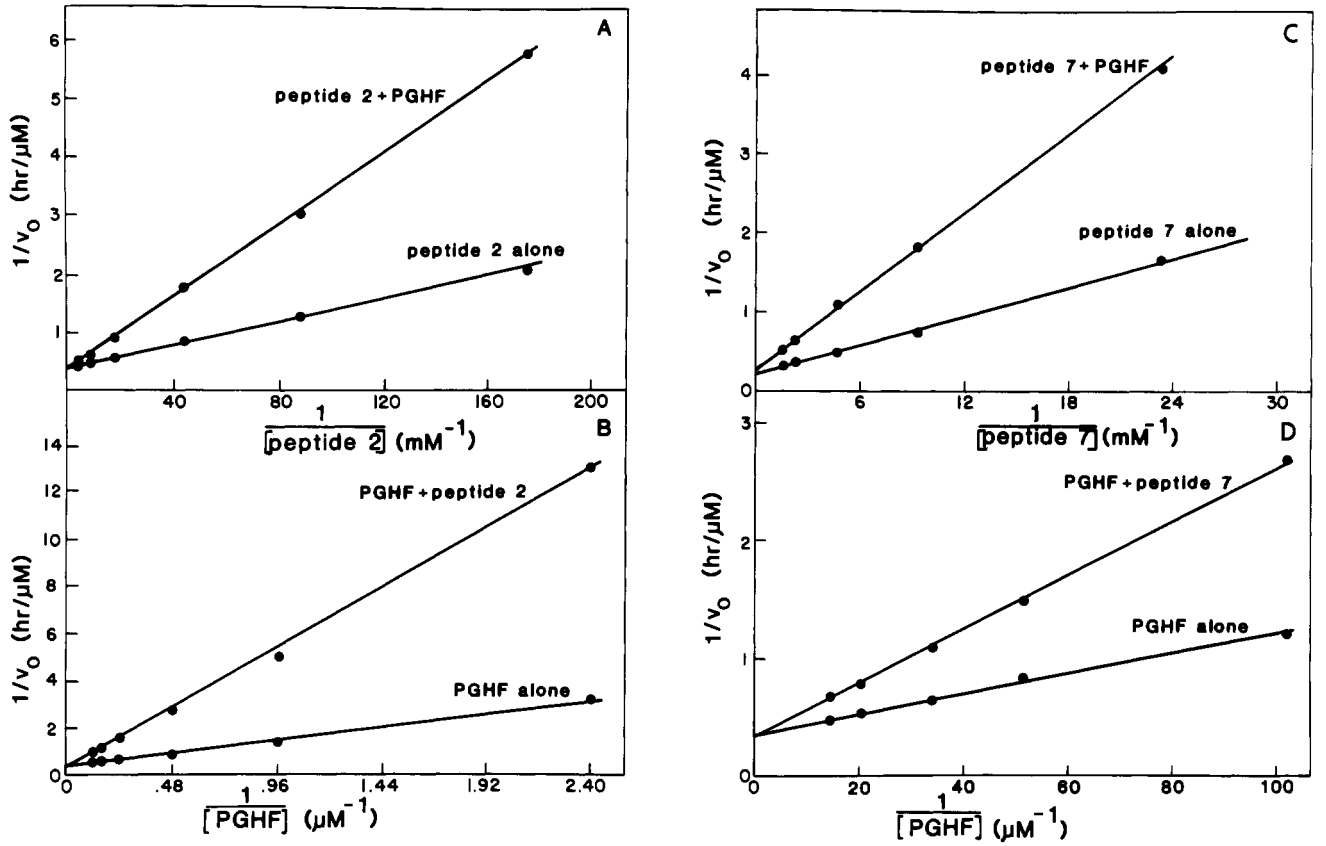

FIGURE 1: Double-reciprocal plots from cross-inhibition studies using peptides and PGHF. Peptide 2 and PGHF were tested for their ability to inhibit each other's acceptor activity. (A) Double-reciprocal plots for the incorporation of [^{14}C]xylose into peptide 2, in the presence or absence of 6.67 μm PGHF. (B) Double-reciprocal plots for the incorporation of [^{14}C]xylose into PGHF, in the presence or absence of 84 μM peptide 2. Peptide 2: K_m 29 ± 1.7 μM; $K_i^{apparent}$ 29 ● 6.6 μM. PGHF: K_m 3.7 ± 0.35 μM; $K_i^{apparent}$ 3.6 ± 0.35 μM. Peptide 7 and PGHF were tested for their ability to inhibit each other's acceptor activity. (C) Double-reciprocal plots for the incorporation of [^{14}C]xylose into peptide 7, in the presence or absence of 4.91 μM PGHF. (D) Double-reciprocal plots for the incorporation of [^{14}C]xylose into PGHF, in the presence or absence of 427 μM peptide 7. Peptide 7: K_m 300 ● 20 μM; $K_i^{apparent}$ 350 ± 45 μM. PGHF: K_m 3.7 ± 0.50 μM; $K_i^{apparent}$ 3.2 ± 0.45 μM. [UDP-xylose] = 50 μM in both peptide experiments.

fragments, 31% of control. These data suggest that the core protein consists of many individual and independent sites for xylose substitution, each of which contains some elements of the proposed consensus sequence.

Kinetic Analysis of Xylosyltransferase Using Artificial Peptides. From the experiments described above, it would appear that the artificial peptides containing the consensus sequence are true analogues of the natural substrate that react with the same site on the enzyme and represent individual xylose-accepting sites on the core protein. Therefore, one peptide, peptide 2, was used as the acceptor substrate for a kinetic analysis of the xylosyltransferase reaction. Initial velocities were measured in steady-state kinetic experiments with UDP-xylose as the varied substrate at several concentrations of peptide 2. The resulting double-reciprocal plots for each substrate are shown in Figures 2 and 3 with their accompanying secondary plots. The steady-state kinetic behavior of bisubstrate enzymes has been analyzed extensively as a means for reaching secure inferences about mechanism (Wong & Hanes, 1962; Cleland, 1963; Cornish-Bowden, 1976; Cleland, 1977). The intersecting initial velocity patterns obtained in the present study are indicative of a single-displacement (also called "sequential") mechanism. Moreover, the nonzero intercepts of the secondary slope plots eliminate rapid equilibrium-ordered forms from consideration.

Inhibition patterns were then used to obtain information concerning the order of substrate entry and product release. When UDP was used as a product inhibitor to determine the order of release of the products, the inhibition was noncompetitive with respect to peptide 2 (Figure 4) and competitive with respect to UDP-xylose (Figure 5). Moreover, PGHF was competitive with the peptide (Figure 1) and noncompetitive with respect to UDP-xylose (data not shown), but this would be the case with a competitive substrate regardless of the order of substrate entry. The ambiguity is partially resolved by comparison of the V_{max} difference between peptide 1 and peptides 2 and 7 (Table I). These values would not be significantly different in a Theorell–Chance mechanism in which the xylosylated peptide was the first product released (Dalziel & Dickinson, 1966).

At this stage of the analysis, two ordered mechanisms remain consistent with all the data: a Theorell–Chance form with the peptide as the leading substrate and UDP as the first product discharged, and a ternary complex form with UDP-xylose as the leading substrate and the xylosylated peptide as the first product.

Some evidence bearing on the distinction between these two mechanisms was sought in the type of mixed alternate substrates experiment originally suggested by Wong and Hanes (1962). If the xylose-acceptor substrate (the peptide) were the leading substrate, providing two different acceptors in the same reaction mixture would cause two different enzyme complexes to be present for reaction with UDP-xylose. If it could be ensured that there were significant fluxes through both pathways, the result in general would be nonhyperbolic UDP-xylose saturation curves for total xylosylation. In contrast, if UDP-xylose were the leading substrate, it would react only with free enzyme in such an experiment, despite the

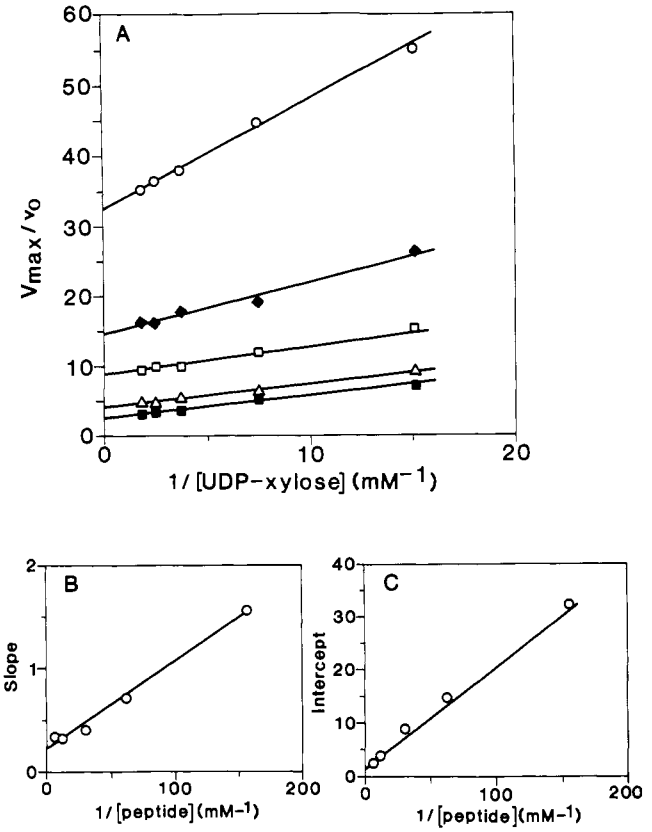

FIGURE 2: Initial velocity pattern with UDP-xylose as varied substrate and secondary plots from substrate covariation. (A) Reciprocal initial velocity plotted versus reciprocal concentration of UDP-xylose. Symbols used correspond to the following concentrations of peptide 2: ○ 6.4 μM, ◆ 16 μM, □ 32 μM, △ 80 μM, ■ 160 μM. (B) Primary slope plotted versus reciprocal concentration of peptide 2. Standard errors are too small to be represented. (C) Primary intercept plotted versus reciprocal concentration of peptide 2. Standard errors are too small to be represented.

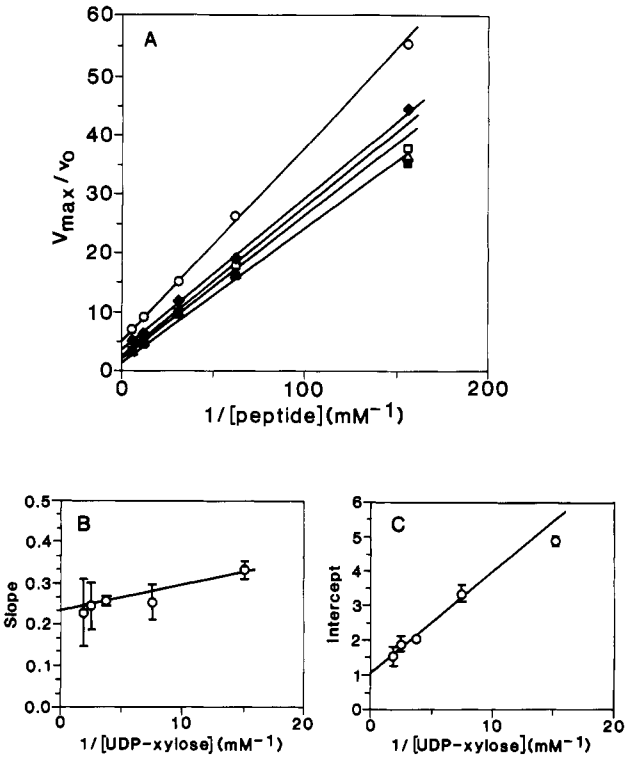

FIGURE 3: Initial velocity pattern with peptide 2 as varied substrate and secondary plots from substrate covariation. (A) Reciprocal initial velocity plotted versus reciprocal concentration of peptide 2. Symbols used correspond to the following concentrations of UDP-xylose; ○ 66 μM, ◆ 133 μM, □ 266 μM, △ 400 μM, ■ 533 μM. (B) Primary slope with standard error plotted versus reciprocal concentration of UDP-xylose. (C) Primary intercept with standard error plotted versus reciprocal concentration of UDP-xylose.

presence of two acceptor substrates, and a normal, hyperbolic saturation curve (linear double-reciprocal plot) would be obtained. Moreover, although the absence of systematic nonlinearity is not in general strong evidence, it has been pointed out that the situation in this type of experiment is better than the general case (Westley, 1969). Both the ability to select alternate substrates that are quite different, on the basis of examination of their kinetic behavior in separate tests, and the ability to establish conditions under which both of the alternate substrates are known unequivocally to contribute to the reaction velocities used for analysis strengthen the inferences available from such an experiment.

Data from a mixed alternate substrates experiment of the type described are presented in Figure 6. Substrate concentrations were chosen such that xylosylation of the PGHF and the peptide in each case contributed about equally to the total xylosylation measured. No systematic nonlinearities were observed in our experiments of this type. Although the case cannot be considered fully settled, it is our opinion that these results significantly favor the mechanism given in Figure 7 as the simplest formal mechanism consistent with all the evidence. The corresponding steady-state rate equation is given in the figure, and the values of the kinetic parameters are presented in Table II.

Computer modeling was carried out by using the rate equation shown in Figure 6 and initially the values for the kinetic constants in Table II. The computer-generated velocity pattern best fitted the experimental data with the following

Table II: Kinetic Parameters of Xylosyltransferase

parameter	value	standard error
V_{max} (μM/h)	47	4.9
$K^{UDP\text{-}xylose}$ (μM)	180	23
$K_m^{peptide\,2}$ (μM)	230	40
K_x (μM²)	6100	1400

values for the kinetic constants: $K_m^{UDP\text{-}xylose} = 275$ μM, $K_m^{peptide\,2} = 200$ μM, and $K_x = 6500$ μM². The good agreement of these values with those derived from the individually fitted lines of the experimental data establishes that the proposed formal mechanism is capable of generating the observed kinetic behavior. The occurrence of a more complex formal mechanism is of course always possible, and some forms with random sequences have not been specifically eliminated.

Discussion

The importance of the proposed xylosylation consensus sequence, Acidic-Acidic-Xxx-Ser-Gly-Xxx-Gly, as a primary signal for the addition of xylose to serines on the core protein of the cartilage CSPG has been investigated by comparison of the acceptor efficiencies (V_{max}/K_m) of synthetic peptides containing this sequence and the deglycosylated core protein, in the in vitro xylosyltransferase assay. The two types of substrates were found to have nearly equivalent acceptor efficiencies and to be competitive inhibitors of each other's acceptor capability with $K_m = K_i^{apparent}$. These results suggest that the artificial peptides represent individual acceptor sites on the core protein and react with the same site on the enzyme with similar reactivity. The kinetic mechanism of the xylosyltransferase reaction was investigated with use of one of the artificial peptides as substrate. The probable mechanism

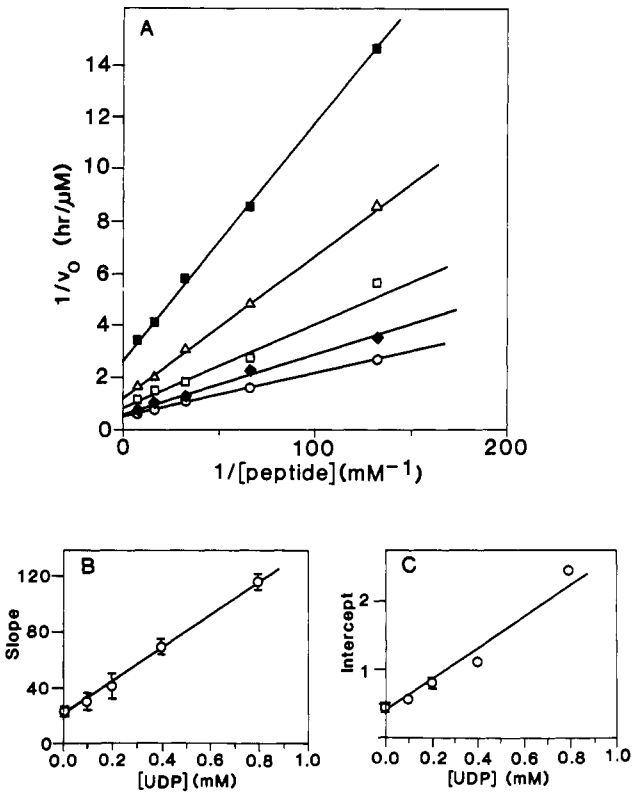

FIGURE 4: Inhibition of UDP with respective to peptide 2. (A) Reciprocal initial velocity plotted versus reciprocal concentration of peptide 2 ([UDP-xylose] = 20 μM). Symbols used correspond to the following concentrations of UDP: ■ 800 μM, △ 400 μM, □ 200 μM, ● 100 μM, ○ 0 μM. (B) Primary slope with standard error plotted versus concentration of UDP. (C) Primary intercept with standard error plotted versus concentration of UDP.

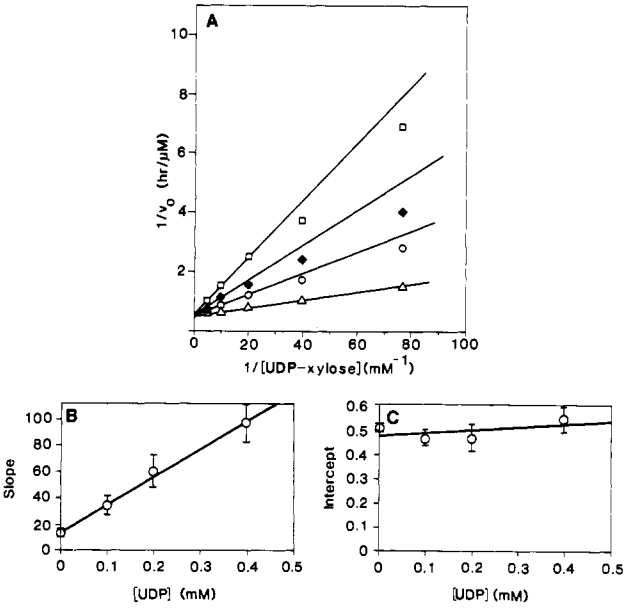

FIGURE 5: Inhibition by UDP with respect to UDP-xylose. (A) Reciprocal initial velocity plotted versus reciprocal concentration of UDP-xylose at the following concentrations of UDP: □ 400 μM, ● 200 μM, ○ 100 μM, △ 0 μM ([peptide 2] = 6 μM). (B) Primary slope with standard error plotted versus concentration of UDP. (C) Primary intercept with standard error plotted versus concentration of UDP. The slope of this secondary plot is not significantly greater than zero.

deduced for the xylosyltransferase reaction is an ordered single displacement, with the substrates entering in the order UDP-xylose and then peptide, and the products released in

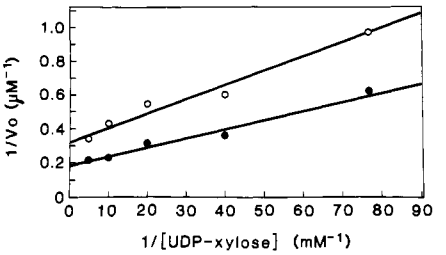

FIGURE 6: Total xylosylation of mixed alternate substrates. Reciprocal initial velocity for total xylosylation of peptide 2 and PGHF is plotted versus reciprocal concentration of UDP-xylose. Symbols correspond to the following: ○ [peptide 2] = 6 μM and [PGHF] = 0.2 μM; ● [peptide 2] = 6 μM and [PGHF] = 0.3 μM.

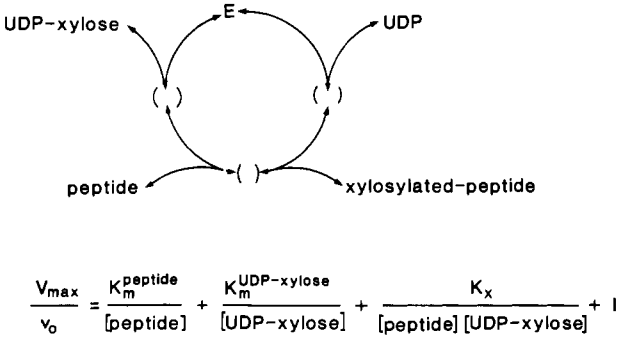

FIGURE 7: Proposed reaction mechanism and rate equation for xylosyltransferase.

the order xylosylated-peptide followed by UDP. This represents the first report of a formal kinetic mechanism for this complex carbohydrate synthesizing glycosyltransferase and the only one for a carbohydrate-to-protein acceptor transferase.

The proposal of a reaction mechanism for xylosyltransferase is also one of the few detailed reports of kinetic studies of glycosyltransferase reactions. In the absence of α-lactalbumin, galactosyltransferase catalyzes the transfer of galactose from UDP-galactose to a monosaccharide, polysaccharide, or a glycoprotein with a terminal *N*-acetylglucosamine residue. This reaction proceeds by an ordered sequential addition of Mn^{2+}, UDP-galactose, and *N*-acetylglucosamine to the enzyme (Kharta et al., 1974). More recently, kinetic characterization of UDP-*N*-acetylglucosamine:α-D-mannoside β-1,2 *N*-acetylglucosaminyltransferase II indicates an ordered, or largely ordered, sequential mechanism with UDP-*N*-acetylglucosamine binding prior to the acceptor substrate (Bendiak & Schachter, 1987). The similarity of these two mechanisms to the one proposed here for xylosyltransferase is that all three are ordered sequential mechanisms in which the nucleotide sugar donor is bound before the acceptor substrate.

Comparison of the efficiencies of the artificial peptides and PGHF also suggests a model of the core protein consisting of many individual and independent sites for xylose substitution, each containing some features of the consensus sequence. The greater efficiency (V_{max}/K_m) of the deglycosylated core protein (PGHF) as compared to that of the artificial peptides containing the consensus sequence, is largely the result of a much lower K_m for PGHF (Table I). When V_{max}/K_m is normalized for number of substitution sites, the efficiencies for these two types of acceptors compare very favorably. This interpretation is supported by the observed decrease in acceptor activity of core protein peptide fragments generated by V8 protease digestion, which disrupts the consensus sequence. Peptide fragments similarly generated by trypsin digestion of PGHF, with no concomitant disruption of the consensus sequence, exhibited an acceptor activity nearly the same as the that of

intact PGHF. One final approach to confirm the hypothesis of independent glycosylation sites would be a demonstration that PGHF at concentrations $\ll K_m$ continues to accept with first-order kinetics even after 50% of the sites have been substituted. However, given the large number of xylose substitution sites on PGHF, such an experiment would be extremely difficult to perform.

The V8 protease experiments also demonstrate the importance of acidic amino acids, N-terminal to the acceptor serine residue, as proposed in the original consensus sequence (Bourdon et al., 1987). These results, along with the recently obtained direct primary sequence for eight glycosylation sites from two different peptide fragments (Krueger et al., 1990a) demonstrate that the same two features of the original consensus sequence are also used in the multiply substituted proteoglycans; although in no instance were they both found around an individual glycosylation site, as in the singly substituted proteoglycans. In fact, the proporton of acceptor sites preceded by acidic residues (six out of a total of eight) closely approximated the decrease in acceptor activity (70%) after treatment with V8 protease. Another intriguing possibility that was revealed by the recent sequencing work is that the presence of multiple substitution sites in close proximity to one another may be advantageous for efficient substitution of all sites by a processive type of enzyme action. Well-established examples of processivity are the DNA polymerases, which provide a fast and efficient method for DNA replication as well as a mechanism for detecting mismatched nucleotides. Given the domain nature of the CSPG core protein and the consequent tight clustering of chondroitin sulfate chains, the issue of processivity or cooperativity between xylose substitution sites is an interesting concept. Studies are underway to address this issue using artificial peptides containing one or more defined substitution sites and then determining the efficiencies of each site alone and together in a single peptide.

Finally, the results reported here clearly demonstrate the utility of artificial peptides in the study of these key reactions involving modification of a protein substrate. In addition to studies designed to address the possibility of a kinetic advantage to the tight clustering of multiple substitution sites in the core protein, the synthetic peptides may prove advantageous in studies to identify and characterize the active site of xylosyltransferase. This approach has been successfully used to identify the RGD-binding domain of the vitronectin receptor (Smith & Cheresh, 1988), and attempts to photoaffinity cross-link peptides to xylosyltransferase are in progress.

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