Human xylosyltransferase I and N-terminal truncated forms: functional characterization of the core enzyme

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Human XT-I (xylosyltransferase I; EC 2.4.2.26) initiates the biosynthesis of the glycosaminoglycan linkage region and is a diagnostic marker of an enhanced proteoglycan biosynthesis. In the present study, we have investigated mutant enzymes of human XT-I and assessed the impact of the N-terminal region on the enzymatic activity. Soluble mutant enzymes of human XT-I with deletions at the N-terminal domain were expressed in insect cells and analysed for catalytic activity. As many as 260 amino acids could be truncated at the N-terminal region of the enzyme without affecting its catalytic activity. However, truncation of 266, 272 and 273 amino acids resulted in a 70, 90 and > 98 % loss in catalytic activity. Interestingly, deletion of the single 12 amino acid motif G²⁶¹KEAISLSRAK²⁷² leads to a loss-of-function XT-I mutant. This is in agreement with our findings analysing the importance of the Cys residues where we have shown that C276A

mutation resulted in a nearly inactive XT-I enzyme. Moreover, we investigated the location of the heparin-binding site of human XT-I using the truncated mutants. Heparin binding was observed to be slightly altered in mutants lacking 289 or 568 amino acids, but deletion of the potential heparin-binding motif P⁷²¹KKVFKI⁷²⁷ did not lead to a loss of heparin binding capacity. The effect of heparin or UDP on the XT-I activity of all mutants was not significantly different from that of the wild-type. Our study demonstrates that over 80 % of the nucleotide sequence of the XT-I cDNA is necessary for expressing a recombinant enzyme with full catalytic activity.

Key words: core enzyme, glycosaminoglycan, heparin-binding site, N-terminal truncated mutant, UDP-xylose, xylosyltransferase I.

INTRODUCTION

Glycosylation is of fundamental biological importance to both prokaryotes and eukaryotes and is catalysed by enzymes forming a large protein family, the glycosyltransferases. These enzymes transfer a sugar moiety from an activated glyconucleotide to an acceptor, which may be a growing oligosaccharide, a lipid or a protein. Glycosyltransferases play a central role in all synthetic processes involving carbohydrates [1–4], and also provide an important technological tool, as they have opened new perspectives in chemoenzymatic synthesis of oligosaccharides [5,6]. Despite the many applications of these enzymes, the precise catalytic mechanism has remained elusive, as the glycosyltransferases share virtually no sequence homology.

Proteoglycans, a large group of glycosylated proteins, are macromolecules composed of GAG (glycosaminoglycan) chains covalently bound to a protein core. The different types of proteoglycans are distinguished by their various core proteins and by the structures of the polysaccharide chains [7]. In GAG biosynthesis, the disaccharide units are formed by alternative monosaccharide addition from corresponding UDP-sugars to the non-reducing end of the elongating chain [8]. The GAGs chondroitin sulphate, dermatan sulphate and heparan sulphate are initiated by the formation of a common carbohydrate sequence, GlcA β 1,3Gal β 1,3Gal β 1,4Xyl β -, bound to specific serine residues in the core protein to form the so-called GAG protein linkage region [9].

XT-I (xylosyltransferase I) is the chain-initiating enzyme involved in the biosynthesis of GAG-containing proteoglycans [10,11]. The enzyme catalyses the transfer of D-xylose from UDP-D-xylose to specific serine residues of the core protein and

is a regulatory factor in chondroitin sulphate biosynthesis [9]. XT-I has a type II transmembrane protein topology, as do all Golgi-associated glycosyltransferases cloned so far. As generally seen for these enzymes, a short cytoplasmic segment of XT-I is connected to a transmembrane domain followed by a large intraluminal domain consisting of a stem region and a catalytic domain.

Comparison of the amino acid sequences of the XT-I from different species shows that this form is highly conserved, but the N-terminus is the most variable region of the enzyme [12]. Recently, we have analysed the importance of the two DXD (Asp-Xaa-Asp) motifs at the N-terminal and the C-terminal region of these glycosyltransferases [13]. We have found that mutations in the D³¹⁴ED³¹⁶ motif did not have any effect on the enzymatic activity, whereas alterations of the D⁷⁴⁵WD⁷⁴⁷ motif resulted in a reduced XT-I activity. Consequently, the comparison of the amino acid sequences and the importance of the DXD motif near the C-terminus raise the question of how many amino acids of the enzyme are necessary for a fully enzymatically active XT-I.

As part of our ongoing studies on XT-I, we have now analysed the functional role of the N-terminal region and examined regions of this enzyme which are responsible for binding to heparin. Thus a number of truncated forms of the enzyme were prepared and characterized. The acquired data are a prerequisite for further structure–function studies on this enzyme.

EXPERIMENTAL

Materials

High Five insect cells (*Trichoplusia ni*, BTI-TN-5B1-4) were purchased from Invitrogen (Groningen, The Netherlands), Insect

Abbreviations used: DTT, dithiothreitol; GAG, glycosaminoglycan; wt, wild-type; XT-I, xylosyltransferase I; rXT-I, recombinant XT-I.

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Figure 1 Schematic representation of the plasmid construction

A schematic representation of the constructs designed for the expression of truncated forms of human XT-I is shown. Sequences coding for the truncated N-terminal parts of XT-I were amplified as described in the Experimental section.

Xpress medium was obtained from Cambrex (Apen, Germany) and *aqua ad injecta* from Braun (Melsungen, Germany). Heparan sulphate, UDP, heparin from porcine intestinal mucosa, heparin-acrylic beads and the Bicinchoninic Acid Protein Assay Kit were purchased from Sigma (Deisenhofen, Germany). Cell culture flasks, serological pipettes and sterile tubes were purchased from Becton Dickinson (Heidelberg, Germany). The vector pMIB-V5-His and the horseradish peroxidase-coupled anti-V5 antibodies were from Invitrogen. Taq polymerase and Hotstar Taq polymerase were from Qiagen (Hilden, Germany). Qiaprep Spin Plasmid kit and the PCR purification kit were also obtained from Qiagen. Precast polyacrylamide gels, buffers and NuPAGE electrophoresis system XCell II Mini-Cell and Blot Module were from Novex (San Diego, CA, U.S.A.). Ultrafiltration cells and PVDF membranes (Immobilon P) were purchased from Millipore (Eschborn, Germany). UDP-[^{14}C]xylose (9.88 kBq/nmol) was obtained from DuPont (Bad Homburg, Germany), 25 mm diameter nitrocellulose discs were from Sartorius (Göttingen, Germany), and the scintillation mixture and liquid-scintillation counter LS5000TD were obtained from Beckman Coulter (Fullerton, CA, U.S.A.). The digital imaging system was from Raytest (Straubenhardt, Germany), and DNA sequencing was performed on an automated DNA sequencing system from PerkinElmer (Foster City, CA, U.S.A.). All other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany).

Plasmid construction of XT-I mutants

PCR amplification and cloning of a soluble form of human XT-I resulting in the vector pCG255 were described previously [13] and used as a template to generate truncated XT-I mutants by PCR.

A construct coding for XT-I lacking the N-terminal cytoplasmic tail, the transmembrane region, parts of the stem region and the C-terminus (see Figure 1) was obtained by PCR amplification using a sense primer containing a HindIII site and nt 553–561 of the XT-I coding region and an antisense primer comprising an EcoRI site and nt 2005–2025. The PCR fragment was subcloned into the HindIII–EcoRI sites of pCG255 to generate [Δ 1–184]XT-I, which harbours the coding region of a truncated XT-I cDNA in frame with the honeybee mellitin secretion signal and a C-terminal V5 epitope for detection. A series of N-terminal truncated constructs was obtained by varying the sense primer and using the same antisense primer. The sequence of the sense primers is indicated in Table 1. Truncated DNA was double-strand sequenced to confirm the reading frame and to ensure that no additional changes were introduced. All other plasmids encoding XT-I mutants were generated by site-directed mutagenesis.

Site-directed mutagenesis

Codons were altered using a method based on the QuickChange site-directed mutagenesis kit. The sequence of the sense and the antisense mutation primers is indicated in Table 1. Mutated DNA was double-strand sequenced to confirm the codon changes or the deletions and to ensure that no additional changes were introduced.

Heterologous expression of truncated XT-I mutants in insect cells

The various mutants were individually expressed in *High Five* insect cells. Transient expression experiments of the truncated XT-I were performed in 3 ml of *High Five* insect cell cultures (2×10^6 cells) seeded in a 60 mm tissue culture plate, transfected

Table 1 Sequences of oligonucleotides

The primers used for amplification of modified constructs of pCG255 are shown. aa, amino acids.

Primer	Oligonucleotide sequence	Generated vector
XT1Hind167U	5'-GATGAGGCAAGCTTACCACCGAGT-3'	Δ1-184aa
XT1Hind244U	5'-GATGAGGCAAGCTTAGGTGAGGTG-3'	Δ1-213aa
XT1Hind385U	5'-GATGAGGCAAGCTTAGGCAAGGAG-3'	Δ1-260aa
XT1Hind403U	5'-GATGAGGCAAGCTTAGCCCTGTCC-3'	Δ1-266aa
XT1Hind421U	5'-GATGAGGCAAGCTTATCCAAGCAC-3'	Δ1-272aa
XT1Hind424U	5'-GATGAGGCAAGCTTAAGCACTGC-3'	Δ1-273aa
XT1Hind427U	5'-GATGAGGCAAGCTTACACTGCCGC-3'	Δ1-274aa
XT1Hind472U	5'-GATGAGGCAAGCTTAGGGCTGCTG-3'	Δ1-289aa
XT1Hind1309U	5'-GATGAGGCAAGCTTAGTGGACTGG-3'	Δ1-586aa
XT1Eco2025L	5'-GTGTGTGAATTCGGCAGTGG-3'	All N-deletion mutants
XTL_K262A_U	5'-AAGTGTGACATCTCAGGCGCGGAGGCC ATC TC-3'	pJD101_K262A
XTL_K262A_L	5'-GAGATGGCCTCCGCGCTGAGATGTACACTT-3'	pJD101_K262A
XTL_E263A_U	5'-GTGACATCTCAGGCAAGCGGCCATCTCTG-3'	pJD102_E263A
XTL_E263A_L	5'-CAGAGATGGCGCCTTGCTGAGATGTCAC-3'	pJD102_E263A
XTL_S266A_U	5'-AAGGAGGCCATCGCTGCCCTGTCCC-3'	pJD103_S266A
XTL_S266A_L	5'-GGGACAGGGCAGCGATGGCCTCCTT-3'	pJD103_S266A
XTL_S269A_U	5'-ATCTCTGCCCTGGCCGTGCTAAGT-3'	pJD104_S269A
XTL_S269A_L	5'-ACTTAGCAGGGCCAGGGCAGAGAT-3'	pJD104_S269A
XTL_R270A_U	5'-CTGCCCTGTCCGCTGCTAAGTCAA-3'	pJD105_R270A
XTL_R270A_L	5'-TTGACTTAGCAGCGGACAGGGCAG-3'	pJD105_R270A
XTL_K272A_U	5'-TGTCCCGTGTGCTGCCAAGCACTG-3'	pJD106_K272A
XTL_K272_L	5'-CAGTGTCTGGACGACGACGGGACA-3'	pJD106_K272A
XTIdelG261K272U	5'-CAGCCCCCTAAGTGTGACATCTCATCCAAGCACTGC-3'	pJD100Δ261-272aa
XTIdelG261K272L	5'-GCAGTGTCTGGATGAGATGTACACTTAGGGGGCTG-3'	pJD100Δ261-272aa
K_722_E_U	5'-CCTGGGTGATGCCGGAAGAAAG TCTTCAAGATCGC-3'	pJD108_K722E
K_722_E_L	5'-GCGATCTTGAAGACTTTTCCGGCATCACCCAGG-3'	pJD108_K722E
K_723_E_U	5'-GGGTGATGCCGGAAGAAAGTCTTCAAGAT-3'	pJD109_K723E
K_723_E_L	5'-ATCTTGAAGACTTCTTCCGGCATCACCC-3'	pJD109_K723E
K_726_E_U	5'-GAAAAAGTCTTCGAGATCGCAAGCCACC-3'	pJD110_K726E
K_726_E_L	5'-GGTGGGCTTGGCATCTCGAAGACTTTTTC-3'	pJD110_K726E
del1762_1782	5'-AGACCTGGGTGATGGCAAGCCACCC-3'	pJD107Δ721-727aa
A_del1762_1782	5'-GGGTGGGCTTGCATCACCCAGGTCT-3'	pJD107Δ721-727aa

with truncated pCG255 vector and incubated for 96 h at 27°C. The culture media containing the recombinant protein were collected and clarified by centrifugation for 5 min at 1500 g. The supernatant was enriched 50-fold by ultrafiltration using Microcon 3000 tubes, and the XT-I-V5 epitope fusion proteins were detected by Western-blot analysis. A stable *High Five*/pCG255-1 insect cell clone expressing rXT-IΔ1-148-V5-His was generated as described elsewhere in detail [14]. At least two vectors encoding each XT-I variant and two independent transfection experiments were performed for the analysis of these mutants in order to ensure reproducibility of the results.

Western-blot analysis

Culture supernatants of transfected *High Five* cells were subjected to SDS/PAGE (4–12 % polyacrylamide) under non-reducing conditions. Separated proteins were blotted on to PVDF membranes and detected with anti-V5 antibodies coupled with horseradish peroxidase at 1:1000 dilution as described previously [12].

Synthesis of recombinant bikunin

Recombinant bikunin was expressed in *Escherichia coli* strain BL21(DE3) as described previously [15]. The purified protein was then used as acceptor in the XT-I activity assay.

Assay for enzyme activity

The method for determination of XT-I activity is based on the incorporation of [¹⁴C]D-xylose with recombinant bikunin as accep-

tor. The reaction mixture for the assay contained in a total volume of 100 μl: 50 μl of XT-I solution, 25 mM Mes (pH 6.5), 25 mM KCl, 5 mM KF, 5 mM MgCl₂, 5 mM MnCl₂, 1.0 μM UDP-[¹⁴C]D-xylose and 1.5 μM recombinant bikunin [16]. After incubation for 1.25 h at 37°C, the reaction mixtures were placed on nitrocellulose discs. After drying, the discs were washed once for 10 min with 10 % (w/v) trichloroacetic acid and three times with 5 % trichloroacetic acid solution. Incorporated radioactivity was quantified after the addition of 3.5 ml of scintillation mixture using a LS5000TD liquid-scintillation counter. The enzyme activity was expressed in units (1 unit = 1 μmol of incorporated xylose/min). The linear range of the XT-I activity assay was determined as 0.02–3.5 m-units/l [16]. To measure within the linear range, samples were diluted with PBS supplemented with 1 % (w/v) human serum albumin. The assay linearity and the dilution procedure were validated using an enriched human XT-I solution, which was prepared as described previously [17].

Commercial preparations were used for the investigation of the influence of effectors on the XT-I activity: heparin from porcine intestinal mucosa, heparan sulphate and UDP. Prior to initiating the reaction, the samples with the enzyme were preincubated for 5 min at 25°C. The XT-I activity was then assayed as described above.

Determination of Michaelis–Menten constant (K_m) and maximal reaction rate (V_{max})

For the determination of the K_m values, various concentrations of the recombinant bikunin were incubated with diluted XT-I

solution and UDP-[¹⁴C]xylose under assay conditions as described above. The recombinant bikunin with a short leader sequence and one xylosylation site per molecule has a molecular mass of 17.52 kDa. K_m and V_{max} values were calculated on the basis of nonlinear regression analysis.

Sensitivity to DTT (dithiothreitol)

Reduction of disulphide bridges was performed by incubation of diluted supernatant from *High Five* insect cells expressing wt (wild-type) or truncated XT-I with increasing concentrations of DTT (0–40 mM) for 10 min at 25 °C. The mixture was assayed under standard conditions as described above.

Measurement of total protein concentration

Protein measurement was determined by the Bicinchoninic Acid Protein Assay Kit using BSA as a standard.

Binding of rXT-I (recombinant XT-I) mutants to immobilized heparin

A 20 µl aliquot of heparin-acrylic beads was washed twice with PBS to remove unbound heparin molecules and resuspended in 20 µl of PBS. Cell culture supernatant (150 µl) containing the XT-I mutants was added and incubated for 1 h at 25 °C under rotation. The beads were then separated by centrifugation at 1000 g for 1 min, the supernatant was removed and the beads were washed twice with 100 µl of PBS. The beads were boiled in a sample buffer [1.00 M Tris/HCl, 1.17 M sucrose, 0.28 M SDS, 2.08 mM EDTA, 0.88 mM Serva Blue G250, 0.70 mM Phenyl Red and 0.10 M DTT (pH 8.5)] for 10 min and the samples were then loaded on to an SDS/PAGE and electrophoresed as described above. The bound rXT-I and N-terminal truncated mutants were then detected after Western blotting using a V5 antibody. For densitometric analysis, the Western blots were then digitized by a computer scanning device and analysed by the Scion Image for Windows software Version 4.02 (Scion Corporation, Frederick, MD, U.S.A.).

Analysis of XT-I amino acid sequences

Homology analysis of the amino acid sequences of the XT-I was carried out with the ClustalX and Genedoc software packages using the following sequences: human XT-I (GenBank® accession number NM_022166) [17], *Mus musculus* XT-I (GenBank® accession number NM_175645), *Rattus norvegicus* XT-I (GenBank® accession number XM_341912) [17], *Pan troglodytes* XT-I (GenBank® accession number CAI28933), *Canis familiaris* XT-I (GenBank® accession number NM_001008718), *Xenopus laevis* XT (GenBank® accession number BC084672), *Tetraodon nigroviridis* XT-I (Ensembl gene GSTENT00021289001), *Drosophila pseudoobscura* XT (GenBank® accession number CAI28925), *Apis mellifera* XT (GenBank® accession number XP_397293) and *Caenorhabditis elegans* XT (GenBank® accession number AJ496235).

Statistical analysis

Results are expressed as the means ± S.D. and statistical analysis was performed using *t* test and the Kolmogoroff–Smirnov test where appropriate. Normality testing for Gaussian distribution of values was performed using the *F*-test. *P* values of 0.05 or less were considered significant.

RESULTS

Alignment of xylosyltransferase sequences of mammals and their orthologues

Using the ClustalX alignment method, multiple sequence alignment of the N-terminus of the reported xylosyltransferases was performed. Figure 2 shows the aligned N-terminal sequences of human XT-I with its mammalian, *Xenopus*, *Tetraodon*, *C. elegans* and insect orthologues, revealing no pronounced similarity within the putative cytoplasmic tail, the transmembrane region and parts of the stem regions. Analysing the stem region, mammals show a high homology in one region consisting of approx. 40 amino acids, in contrast with insects and *C. elegans*. *In vivo*, this region may probably play a role in generating a soluble form of the enzyme, because the cleavage site for a potential protease has not yet been identified. In common with the beginning of the highly homologous region in mammals, we postulated the onset of the catalytic domain between Ser²⁶⁰ and Ser²⁶⁶.

XT-I of human, chimpanzee, mouse, rat and dog showed 99–91 % identity. Comparing the mammal XT-I with insect XT, only 31–35 % amino acid identity was found. *Xenopus* and *Tetraodon* sequences showed a homology of 77 and 63 % to human XT-I.

Expression of soluble human XT-I and N-terminal truncated mutants of XT-I

The role of the N-terminus of the human XT-I for full catalytic activity was investigated by generating constructs with sequential N-terminal deletions (Figure 3). Mutant enzymes were engineered which were truncated at Lys¹⁸⁴, Pro²¹³, Ser²⁶⁰, Ser²⁶⁶, Lys²⁷², Ser²⁷³, Lys²⁷⁴, Leu²⁸⁹ and Ile⁵⁶⁸ as indicated in Figure 1. The initiating methionine and the secretion signal from the vector were retained in each case. These constructs were termed [Δ1–184]XT-I, [Δ1–213]XT-I, [Δ1–260]XT-I, [Δ1–266]XT-I, [Δ1–272]XT-I, [Δ1–273]XT-I, [Δ1–274]XT-I, [Δ1–289]XT-I and [Δ1–568]XT-I respectively, where the numbering refers to the amino acid residues deleted. Furthermore, constructs lacking the amino acids 261–272, [Δ261–272]XT-I, and with single amino acid substitutions at positions 262, 263, 266, 269, 270 and 272 were generated. The mutated enzymes of XT-I were expressed in insect cells, and the catalytic profiles were compared with that of the wt XT-I enzyme.

A soluble form of the human XT-I lacking the cytoplasmic tail and transmembrane domain was expressed in *High Five* insect cells with high activity as described elsewhere [12,13]. Following SDS/PAGE, a single sharp band was detected on Western blots using antibodies specific for the V5 epitope expressed at the C-terminal end of the recombinant protein (Figure 3). We investigated N-terminal truncated forms of rXT-I in disulphide bond formation and in catalytic activity by constructing mutants. All mutants were found to be expressed in insect cells at similar levels to the wt protein and were characterized by SDS/PAGE. When the proteins were analysed under reducing conditions, the rXT-I and all truncated mutants gave a single band of expected molecular mass (Figure 3).

Characterization of rXT-I and truncated mutants for catalytic activity

The activities of the mutant proteins were determined in XT-I activity assays as described in the Experimental section. rXT-I was always expressed in parallel with the mutants as a positive control. In all experiments, the wt XT-I and truncated mutants were expressed at comparable levels. Five mutants, lacking 272 amino acids or more, showed a complete loss of function (Figure 3). The Δ1–266 mutant resulted in a loss of enzymatic activity of

Figure 2 Alignment of xylosyltransferase sequences

The N-terminal region of the coding sequences of ten different species are shown including human (human_XTI), mouse (mus_m.), rat (rattus_n.), chimpanzee (pan_t.), dog (canis_f.), frog (xenopus_l.), pufferfish (tetraodon), *Drosophila*, honeybee (apis_m.) and worm (c. elegans). Introduced gaps are shown by dashes. Black boxes indicate identical amino acids in all proteins. Highly conserved clusters are indicated in grey: dark grey boxes indicate amino acids with 80–99% identity and light grey boxes amino acids with 60–79% identity among the species investigated. The potential transmembrane region is marked by a line. The first amino acid of the recombinant soluble rXT-IΔ148 enzyme expressed in the present study and three different truncations are marked by asterisks.

more than 70%, indicating the necessity of these residues for enzymatic activity. N-terminal truncated enzymes lacking the first 260 amino acids (Δ1–260) showed 100% activity compared with the wt XT-I.

Taken together, these results suggest that the loss of 260 amino acids at the N-terminus of the protein is not critical for the activity of rXT-I. Interestingly, truncated enzymes lacking only 12 amino acids more (Δ1–272) showed a nearly complete loss of function, indicating a critical structure feature in this region. The kinetic analysis of the wt secretory XT-I and mutants revealed both increased K_m values and a reduced maximal velocity for the mutants [Δ1–266]XT-I and [Δ1–272]XT-I. The apparent kinetic parameters for wt and mutant XT-I are presented in Table 2. In order to further analyse the role of these 12 amino acids, GKEAISALSRK, in the enzymatic function, we generated a soluble XT-I mutant lacking this 12 amino acid motif, [Δ261–272]XT-I. Interestingly, deletion of this motif leads to a complete loss of XT-I activity although the protein was expressed at com-

parable levels. Consequently, we generated mutants with each of the non-aliphatic amino acids being changed to alanine. Analysis of the enzyme kinetics of these mutants did not reveal any significant changes in K_m or V_{max} , with the exception of [R270A]XT-I where V_{max} was reduced by at least 50% in comparison with the wt (Table 2). The XT-I variants [K722E]XT-I, [K723E]XT-I and [K726E]XT-I which share single amino acid substitutions in the potential Cardin–Weintraub heparin-binding motif had enzymatic properties comparable with rXT-I (results not shown). However, deletion of this six amino acid motif in the [Δ721–726]XT-I leads to a complete loss of function.

rXT-I enzyme activity under reducing conditions

In previous experiments, we have shown that the addition of DTT to rXT-I produced a loss of activity of approx. 95% [12]. In the present study, we wanted to investigate whether the inactive truncated mutants could be activated with DTT and to determine

Figure 3 XT-I activity of rXT-I and the truncated mutants

Top: relative activities of wt XT-I and N-terminal truncated mutants. The bar chart indicates the percentage XT-I activity relative to the rXT-I wt with error bars representing the S.D. Bottom: Western blots of 50× concentrated media containing wt XT-I (rXT-I) and mutants under reducing conditions (lane M, multicolour prestained molecular mass standard). Proteins were visualized using anti-V5 antibodies.

Table 2 Kinetic constants for the wild-type rXT-I and its N-terminal truncated mutants

Michaelis–Menten constants (K_m) and maximal reaction rates (V_{max}) of rXT-I and mutants with recombinant bikunin as acceptor protein. XT-I activity was assayed as described in the Experimental section. Enzyme activity is expressed as μmol of [^{14}C]xylose incorporated into bikunin per min. Results are the average of two independent assays; n.d., not determined.

Mutant	K_m (μM)	V_{max} (pmol/h)	V_{max}/K_m (pmol · h ⁻¹ · μM^{-1})
wt XT-I	0.9	841	934
[Δ 1–184]XT-I	0.9	990	1100
[Δ 1–213]XT-I	1.4	930	664
[Δ 1–260]XT-I	1.4	840	600
[Δ 1–266]XT-I	5.0	540	108
[Δ 1–272]XT-I	5.4	640	118
[Δ 1–273]XT-I	n.d.	n.d.	–
[Δ 1–274]XT-I	n.d.	n.d.	–
[Δ 1–289]XT-I	n.d.	n.d.	–
[Δ 1–568]XT-I	n.d.	n.d.	–
[K262A]XT-I	1.1	1428	1298
[E263A]XT-I	1.3	1667	1282
[S266A]XT-I	1.3	1111	855
[S269A]XT-I	1.2	909	758
[R270A]XT-I	1.1	588	534
[K272A]XT-I	1.3	1429	1099
[Δ 261–272]XT-I	n.d.	n.d.	–
[Δ 721–726]XT-I	n.d.	n.d.	–

the sensitivity to DTT of the active mutants. We incubated the mutant enzymes with increasing concentrations of DTT and analysed them for activity. None of the inactive mutants tested could be activated with DTT. The addition of 0.62 mM DTT inhibited the [Δ 1–184]XT-I mutant by 80 %, comparable with the wt XT-I protein (Figure 4). [Δ 1–213]XT-I, [Δ 1–260]XT-I and

Figure 4 Effect of DTT on rXT-I enzyme activity

The inhibitory effect of DTT on the activity of wt enzyme and on Δ 1–184, Δ 1–213, Δ 1–260 and Δ 1–266 expressed in a parallel experiment is indicated. None of the tested mutants could be activated with DTT.

[Δ 1–266]XT-I showed an increased inhibition. It is noteworthy that the tested mutants were not equally as sensitive to DTT inactivation as the wt protein. Furthermore, the addition of 2.5 mM DTT reduced the enzyme activity of wt and all active truncated enzymes of XT-I to approx. 5 %.

Inhibition by heparin and binding of rXT-I to immobilized heparin

Human XT-I has a high affinity for heparin, and we have previously shown that the enzyme strongly binds to the heparin matrix during heparin affinity chromatography [14,18,19]. Thus we used the truncated XT-I mutants generated to investigate the inhibitory effects of and the binding characteristics to heparin. All mutants tested could be strongly inhibited by heparin at concentrations ranging from 1 to 100 i.u./ml (results not shown). At 1 i.u./ml, inhibition of 95 % was observed using the active mutants and rXT-I. No significant differences between any of the mutants and the rXT-I wt were observed. Binding of rXT-I and the N-terminal truncated mutants to heparin was investigated using heparin-acrylic beads. Heparin beads were incubated with the crude enzyme preparations and, after washing, the amount of enzyme bound to the beads was visualized using Western blotting. As expected, most of the rXT-I enzyme present in the sample was bound to the heparin beads and was not released by washing steps. Similar results were obtained by analysing the truncated mutants (Figure 5), but with slightly reduced binding capacities using the mutants [Δ 1–289]XT-I and [Δ 1–568]XT-I. Interestingly, the mutants [K722E]rXT-I, [K723E]rXT-I and [K726E]rXT-I with the essential basic amino acids of the potential Cardin–Weintraub heparin-binding motif being changed to acidic residues did not show any altered heparin binding using this assay. Even deletion of the whole motif P⁷²¹KKVFKI⁷²⁷ did not significantly alter the heparin binding properties, indicating only little influence of these amino acids on the heparin binding in rXT-I (Figure 6).

Analysing the heparin-binding site of rXT-I

Analysis of the structural features of many known heparin-binding proteins has revealed the presence of conserved motifs, through

Figure 5 Binding of rXT-I mutants to immobilized heparin

Heparin-acrylic beads were incubated with the samples, centrifuged, washed, boiled in sample buffer and electrophoresed as described in the Experimental section. Detection of soluble rXT-I and mutants bound to the beads was performed with anti-V5 antibodies. Most of the enzyme present in the sample was bound to the heparin beads. Lane 1, control (equal amounts of protein were added to each control lane and to the heparin beads). Lane 2, boiled heparin beads.

Figure 6 Binding properties of the mutants K722E, K723E, K726E and [Δ721–726]XT-I to heparin

The heparin binding properties of XT-I mutants with alterations in the potential Cardin–Weintraub heparin-binding motif P⁷²¹KKVFK⁷²⁷ were investigated. Heparin-acrylic beads were incubated with the samples, centrifuged, washed, boiled in sample buffer and electrophoresed as described in the Experimental section. Detection of soluble rXT-I and mutants bound to the beads was performed with anti-V5 antibodies and signal intensity was quantified by densitometric analysis. Means $\pm$ S.D. for three experiments are shown.

which binding has been postulated to occur. In order to investigate whether the heparin-binding site exists in the tertiary structure of XT-I or in consensus sequences, we investigated the binding of rXT-I to heparin beads under reducing conditions with 2.5 mM DTT. Additionally, we analysed a sample after incubation at 80 °C for 5 min for binding to heparin beads. The sample that was added with DTT showed no significantly different binding characteristics to heparin beads compared with the untreated control.

Figure 7 Binding of rXT-I to immobilized heparin under reducing and non-reducing conditions

Recombinant XT-I (wt) was incubated with heparin beads under reducing and non-reducing conditions. The amount of rXT-I bound to heparin beads under reducing conditions with 2.5 mM DTT (lane 2) and after heat denaturation (lane 3) is shown. Lane 1: control; equal amounts of protein were added to the control lane and to the heparin beads.

Efficient binding of rXT-I to the heparin-coated beads was shown even under denaturing conditions (Figure 7, lane 3).

Effect of mutations on binding to UDP

UDP is a competitive inhibitor of many glycosyltransferases [15,16] including the human XT-I. Therefore we analysed the role of the truncated XT-I mutants on the nucleotide sugar binding. The inhibition of the enzymatic activity by UDP at different concentrations was investigated for the active XT-I mutants generated. The addition of 0.25 mM UDP inhibited the wt XT-I activity by 98 %, whereas the addition of 1.5 mM UDP resulted in a loss of activity of more than 99 %. After the addition of 0.25 mM UDP, all tested mutants showed a similar inhibition by approx. 98 %. We found no significant influence on the reduction of the relative XT-I activity after addition of UDP.

Effect of heparan sulphate on truncated mutant enzymes

Inhibition of the enzymatic activity of wt XT-I enzyme by heparan sulphate has been shown in previous studies [20]. In order to investigate a potential inhibitory effect of GAGs on the enzymatic activity of truncated mutant proteins, heparan sulphate was added to samples, and the XT activity was determined. The effector concentrations used covered a range from 10 to 1000 μ g/ml. Large amounts of heparan sulphate (1000 μ g/ml) revealed up to 80 % inhibition of wt XT-I activity. Furthermore, small amounts did not have any influence on activity. However, all N-terminal truncated mutant enzymes were as equally sensitive to heparan sulphate inhibition as the wt protein.

DISCUSSION

The protein domain structure proposed several years ago for glycosyltransferases indicates that the C-terminal portion of these enzymes contains their catalytic domain. The results we have obtained in the present study demonstrate that approx. 20 % of the N-terminal amino acids of the human XT-I sequence are not required for enzyme activity. The [Δ1–266]XT-I mutant and all mutants lacking more amino acids at the N-terminus studied in this paper elicit a reduced activity with respect to the wt recombinant enzyme, whereas the apparent K_m values are only slightly different. The sharp reduction in the activity of this mutant indicates that the removal of only six amino acids somehow affects the active site structure. Δ1–266 and Δ1–272 are localized in front of a region of approx. 300 amino acids with a putative

involvement in the binding of UDP-sugar donors [21]. The onset of these 300 amino acids is localized after the first DXD motif at the position 314–316 and is, therefore, completely maintained in inactive mutants also. The great loss of function between mutant $\Delta 260$ and $\Delta 266$ of approx. 70 % indicates that the loss of catalytic activity does not depend on alterations in the substrate-binding site.

Previously, we have shown that mutations in the N-terminal located cysteine residues have different influences on the XT-I enzyme activity. The C276A mutant resulted in a loss of enzymatic activity of approx. 91 %, whereas the C257A mutant retained approx. 55 % activity. When truncated mutants were tested for activity, our results demonstrate that the amino acids 260–272 are critical for the production and maintenance of an active conformation of the XT-I. Lacking 266 N-terminal amino acids resulted in a loss of activity of 70 %, indicating the critical role of this region, which is probably due to structural modifications at the active site which do not affect the substrate binding.

In order to gain further information about the relevance of this 12 amino acid motif G²⁶¹KEAISLSRAK²⁷² for the enzyme activity, XT-I variants with each of the non-aliphatic amino acids being changed to alanine were generated. Our investigations revealed that a single amino acid within this motif is not responsible for the crucial role of this motif in the enzyme activity. The kinetic analysis revealed that only the R270A alteration resulted in a reduced $V_{\max}$ value with a K_m similar to the wt. As our analysis was limited to changes of the non-aliphatic amino acids in this motif, a significant role of the unchanged small apolar residues alanine, glycine, leucine and isoleucine cannot be completely excluded. Interestingly, deletion of the whole 12 amino acid motif leads to a complete loss of XT-I activity, indicating that this motif is important for the enzyme activity, possibly being necessary for the proper protein conformation or the structural orientation of the adjacent cysteine residue at position 276. Our previous studies have already shown that this cysteine residue is important for the XT-I activity as alterations of Cys²⁷⁶ resulted in loss-of-function mutants [12], opening the possibility that the 12 amino acid motif may be required to achieve correct pairing of cysteine residues.

The alignment of the amino acid sequences from a variety of species revealed that definite motifs are evolutionarily highly conserved with a highly variable N-terminus region (Figure 2). In the present study, we have tested truncated mutants lacking amino acids in variable and highly conserved regions. We could demonstrate that the beginning of a common homologous region in position 259 is also critical for enzymatic activity. The loss of 12 amino acids in this region resulted in a nearly complete loss of function in enzyme activity, indicating the necessity of these residues in retaining a catalytically active structure of XT-I.

We investigated the role of the N-terminus in catalytic activity by constructing mutants in which the N-terminal domain was individually truncated. We were able to produce equal levels of the wt XT-I and truncated mutant XT-I forms in the *High Five* insect expression system. The fact that the truncation of the enzyme in variable sequence regions has little effect on the kinetics of XT-I suggests that the residues tested may not be directly involved in substrate binding.

To analyse the active conformation of XT-I, we investigated the consequences of DTT treatment, i.e. whether the inactive truncated mutants could be activated with DTT and the sensitivity to DTT of the active mutants. We incubated the mutant enzymes with increasing concentrations of DTT and analysed them for activity. Interestingly, the different mutants were not equally sensitive to the reagent. Enzymes lacking 260 and 266 amino acids showed an increased effect on DTT treatment, probably due

to their higher amount of truncation resulting in a destabilized protein structure.

Furthermore, we used the truncated XT-I mutants generated to investigate the inhibitory effects of and the binding characteristics to heparin. Heparin is a potent inhibitor of the human XT-I, and the enzyme strongly binds to a heparin matrix during heparin affinity chromatography [14,18,19]. In the present study, we could demonstrate that the mutants tested could be strongly inhibited by heparin, and the sensitivity to the reagent was equal for all truncated mutants compared with the wt XT-I. We used Western blotting to visualize the amount of enzyme bound to heparin beads. Taken together, these experiments revealed that all mutants were efficiently bound to immobilized heparin, but with slightly different binding properties when analysing the mutants lacking 289 and 568 amino acids (Figure 5). This indicates that the truncation does not induce a misfolding of the heparin-binding site. Probably, not only one binding site mediates the binding of XT-I to heparin. Cardin and Weintraub [22] identified two clusters of basic charge in known heparin-binding proteins in which amino acids tend to be arranged in the patterns XBBXB or XBBBXXBX, where 'B' represents an amino acid with basic charge, usually arginine or lysine, and 'X' represents an uncharged or hydrophobic amino acid. The human XT-I did not show such a consensus sequence, but we have found a similar sequence with the consensus sequence XBBXXBX in position Pro⁷²¹–Ile⁷²⁷, which exists in all truncated mutants. In order to evaluate the significance of this P⁷²¹KKVFKI⁷²⁷ motif for the XT-I–heparin interaction, we generated XT-I variants where the basic lysine residues were changed to glutamic acid. Furthermore, the complete Cardin–Weintraub consensus sequence in position Pro⁷²¹–Ile⁷²⁷ was deleted. Interestingly, neither an altered heparin inhibition nor a reduced binding to heparin was observed using these XT-I mutants. This lets us conclude that the P⁷²¹KKVFKI⁷²⁷ motif is in fact not the primary binding site for heparin and that other positions in the amino acid sequence of XT-I, which do not follow the Cardin–Weintraub rules, mediate the interaction with heparin. It is noticeable that many short clusters of basic amino acids are scattered throughout the sequence of XT-I. This leads to the speculation that the strong binding of the enzyme to heparin depends on a multiplicity of basic clusters in the sequence and not on only one binding site. Further experiments, and probably also the crystal structure, are necessary to localize the exact heparin-binding site of the human XT-I. It is noteworthy that deletion of this seven amino acid motif P⁷²¹KKVFKI⁷²⁷ leads to a nearly complete loss of enzymatic activity probably due to conformational changes induced by the omission of these amino acids. They are located close to the C-terminal D⁷⁴⁵XD⁷⁴⁷ motif, which has previously been demonstrated to be critical for the XT-I activity [13]. These results clearly support the importance of the C-terminal region of the XT-I for the enzymatic activity.

Reflecting on the membership of XT-I in the glycosyltransferase family 14, it is a matter of speculation as to the role of regions of the xylosyltransferase which are not homologous with the other family 14 members. The highly variable N-terminal regions of the xylosyltransferases from different species all share a common PXCD/E motif, which, according to preliminary data from Wilson [21], is necessary for catalytic activity of the *Drosophila* xylosyltransferase. This motif is also common in the human XT-I sequence, and using the N-terminal truncated mutants, we have shown that lacking of the first motif P²⁵⁵–D²⁵⁸ did not have any influence on the enzyme activity of human XT-I. Additionally, we have demonstrated that the C542A mutation in the second motif P⁵⁴⁰–D⁵⁴³ resulted in a loss of activity of approx. 70 %. These results indicate that the first motif is not directly

involved in substrate binding or in achieving an enzymatically active human XT-I. The second motif could probably function in catalytic activity. However, this remains to be elucidated in further experiments.

In the present study, we could demonstrate that the highly variable region of the N-terminus is not required for enzyme activity, nor is it involved in UDP, heparan sulphate or heparin binding. The relevance of the short cytosolic domain for probable translocation remains to be elucidated.

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