Effect of C-domain N-glycosylation and deletion on rat pancreatic α -amylase secretion and activity

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Even though all animal α -amylases include glycosylation sequons (Asn-Xaa-Thr/Ser) in their sequences, amylases purified from natural sources are not quantitatively glycosylated. When wild-type rat pancreatic α -amylase, which contains two glycosylation sequons, was expressed in animal cell lines the protein displayed a very low rate of glycosylation (approx. 2%), even after Brefeldin A treatment to increase the contact with the glycosylation machinery. Site-directed mutagenesis of the first glycosylation sequon (Asn⁴¹⁰ → Gln) resulted in 90 % of the protein being glycosylated at the second glycosylation sequon (Asn⁴⁵⁹). Mutation of the second sequon completely inhibited glycosylation. In order to ascertain if the interference in the glycosylation of Asn⁴⁵⁹ that was eliminated by the Asn⁴¹⁰ → Gln mutation could be due to the position of the asparagine residue in the Cys⁴⁴⁸-Cys⁴⁶⁰ disulphide bridge, these cysteine residues were mutated to serine residues. The resulting mutant was found to be 100 % glycosylated. All mutants with mutations in the C-domain had specific activities identical

to that of the wild-type enzyme, indicating that enzymic activity is independent of the structure and modification of the C-terminal domain. To further test the independence of the C-domain with respect to the two N-terminal domains of the protein, which harbour the catalytic site, the last seven of the ten β -strands that make up the β -sandwich configuration of the domain were deleted. The truncated protein was not secreted from cells and all enzyme activity was destroyed. These observations show that Asn⁴⁵⁹ is the only site that can be glycosylated in wild-type amylase, and confirm the relative independence of the C-terminal domain of α -amylase with respect to enzyme activity. In addition, they also establish that the C-terminal domain is absolutely essential for the correct post-translational folding of the enzyme that is responsible for its activity and allows for its secretion.

Key words: exocrine pancreas, folding, glycohydrolase, intracellular transport, zymogen.

INTRODUCTION

Amylase is the predominant pancreatic exocrine secretory-granule zymogen. Animal α -amylases consist of three structural domains called A, B and C. The A-domain, which is arranged in a (β/α)₈-barrel structure, characteristic of numerous glycan hydrolase families, is the largest and forms the catalytic domain. The B-domain forms a large loop that protrudes from the A-domain, while the C-domain is only loosely associated with the other two domains and is of unknown function. In both the well-characterized pig and human enzymes the C-domain is arranged in a β -sandwich structure [1,2]. In rat pancreatic α -amylase, a 494 residue protein with a molecular mass of 55 kDa, the A-, B- and C-domains are composed of amino acids 1–115+183–402, 116–182 and 403–494 respectively.

With few exceptions [3–5] α -amylase purified from animal sources has always been described as being a non-glycosylated 55000 Da polypeptide, primarily because the molecular mass of the purified protein corresponded to that predicted from the amino acid composition [6]. However, glycosylation sequons (Asn-Xaa-Thr/Ser-Xaa) are present in all animal α -amylase sequences, suggesting that the protein is a potential target of the glycosylation machinery. There are one or two of these sequons per amylase, depending on the species in question, and they are always located at the C-terminus of the protein in the C-domain. The amino acid sequences of pancreatic α -amylases are highly

conserved, with the rat and human enzymes being 85 % identical (89 % similar). In addition, each of these enzymes possesses two glycosylation sequons located at identical positions in the sequences. The two rat glycosylation sequons are located at positions 410 (Asn⁴¹⁰-Gly-Ser-Asn) and 459 (Asn⁴⁵⁹-Cys-Thr-Gly) [7]. According to studies on the influence of the Xaa residues in the glycosylation sequon on the efficiency of glycosylation, the glycine and the asparagine of the rat Asn⁴¹⁰ sequon should favour N-glycosylation [8]. Because the Asn⁴⁵⁹ sequon of the rat enzyme is trapped in a disulphide bridge between Cys⁴⁴⁸ and Cys⁴⁶⁰, and the fact that Cys⁴⁶⁰ is at the first Xaa position in the sequon, it is likely that this structural constraint has a negative impact on glycosylation at this site [9].

Though most non-cytoplasmic proteins are glycosylated, the significance of glycosylation is not always fully understood. Some of the structural effects of glycosylation are now emerging. One of these roles is in protein folding and quality control during biosynthesis and post-translational processing [10]. Another role of glycosylation is in the functioning of mature proteins, for example in biological recognition at the cell surface. In the case of α -amylases, any function of glycosylation on the enzymic activity of the mature protein is quite unlikely since the non-glycosylated form of amylase has been proven to be fully active. However, it is remarkable that all glycosylation sequons of animal α -amylases are located in the C-domain, a domain of unknown function. One of the roles proposed for this domain is

Abbreviations used: BFA, Brefeldin A; DMEM, Dulbecco's modified Eagle's medium; DMEM⁻, DMEM without methionine and cysteine; Endo H, endo- β -N-acetylglucosaminidase H; ER, endoplasmic reticulum; FBS, fetal bovine serum; MDCK, Madin–Darby canine kidney; OST, oligosaccharyl transferase; PNGase F, peptide N-glycosidase F; RIPA, radioimmunoprecipitation assay.

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to help stabilize the $(\beta/\alpha)_8$ -barrel of the A-domain [11]. Another function suggested by Qian et al. [12], who characterized a disaccharide binding site at the interface of the C- and A-domains, would be to serve as an anchor for long chain substrates on their way to the active site.

In the present study we examine the behaviour of the two glycosylation sequons, both independently and together, using site-directed mutagenesis of their asparagine residues. We also examined if increasing the contact between the protein and the glycosylation machinery of the endoplasmic reticulum (ER) would increase the rate of glycosylation, thereby explaining the observed low level of glycosylation of native α -amylases. The structural constraint that the Cys⁴⁴⁸-Cys⁴⁶⁰ disulphide bridge constitutes for glycosylation at Asn⁴⁵⁹ was assessed by mutating both cysteine residues. Finally, to gather more clues on the possible role of the α -amylase C-domain, seven of the ten β -strands that make up the domain were deleted. The enzymic activity of all mutants was determined. The present study has allowed us to gather information on the structural constraints in the C-domain that control N-glycosylation, and to evaluate the importance of the domain in the normal folding of this protein that leads to its secretion and its enzymic activity.

EXPERIMENTAL

Materials

All laboratory reagents were purchased from Sigma unless otherwise indicated. Polyclonal antibody against α -amylase was the same as that used in previous studies [13]. Cell culture media, antibiotics and other supplements were purchased from Life Technologies.

DNA mutagenesis and plasmid construction

All DNA manipulations were performed according to state-of-the-art molecular biology procedures. The partial rat α -amylase cDNA (pcXP100 and pcXP38) was a gift from Dr R. J. MacDonald (University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.) [7]. The five missing codons, preceded by a consensus Kozak sequence, were added to the 5' end of the cDNA by oligonucleotide-dependent mutagenesis. The nucleotides added for the initiation of translation and the first residues of the protein encoded the amino acid sequence MVKFFL (single-letter amino-acid notation) as observed in both the human pancreatic [14] and mouse salivary [15] α -amylases, except for a valine codon added at position 2 so as to create an *NcoI* restriction site. The wild-type and mutant cDNAs were all subcloned into the eukaryotic expression vector pRc/CMV1 (Invitrogen). The two asparagine residues that are targets of glycosylation in the two sequons were mutated to glutamine by oligonucleotide-dependent site-directed mutagenesis. Deletion of the 65 C-terminal residues was performed by inserting a stop codon in the amylase gene after the codon for Asn⁴²⁹ (resulting in $\Delta C\beta 4$ -10). All mutations and constructions were ultimately verified by DNA sequencing.

Cell culture and transfections

Madin-Darby canine kidney (MDCK), COS-1 and AtT20 cells were a gift from Dr G. Boileau (Department of Biochemistry, Faculty of Medicine, University of Montréal, Montréal, QC, Canada). All cell transfections were performed using LIPO-FECTAMINE™ (Amersham Biosciences) according to the manufacturer's protocol. Maintenance of cells and routine cell culture were performed at 37 °C in a humidified atmosphere with 5% CO₂. The stably transfected pools of MDCK cells were cultured

in Dulbecco's modified Eagle's medium (DMEM)/DMEM-F12 (1:1, v/v), 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin G, 100 µg/ml streptomycin sulphate, 0.25 µg/ml amphotericin B and 300 µg/ml G418. Stable MDCK transfectants were obtained by selection with G418 at 400 µg/ml for 2–3 weeks. COS-1 cells were cultured in DMEM supplemented with 10% (v/v) FBS, 100 units/ml penicillin G, 100 µg/ml streptomycin sulphate and 0.25 µg/ml amphotericin B. The stably transfected pool of AtT20 cells was cultured in DMEM, 10% (v/v) fetal calf serum, 10% (v/v) horse serum and 2% (v/v) NU serum™ (Collaborative Biomedical Products, Becton Dickinson Labware, Bedford, MA, U.S.A.), supplemented with 100 units/ml penicillin G, 100 µg/ml streptomycin sulphate, 0.25 µg/ml amphotericin B and 300 µg/ml G418.

Metabolic labelling, immunoprecipitation and quantification of radiolabelled proteins

AtT20 cells stably expressing wild-type rat pancreatic α -amylase were used to assess both the level of glycosylation of the protein and the effects of tunicamycin and Brefeldin A (BFA) on the level of glycosylation. Cells were plated in 6-well culture dishes in complete medium and grown to 80% confluence. The cells were then washed in PBS and treated overnight with 10 mM butyrate in 1 ml of synthetic medium (DMEM containing 5.5 µg/ml insulin, 17.5 µg/ml transferrin, 2 µg/ml ethanolamine, 100 µg/ml soya bean trypsin inhibitor, 10 µg/ml aprotinin, 100 units/ml penicillin G, 100 µg/ml streptomycin sulphate and 0.25 µg/ml amphotericin B). The overnight medium was replaced by 1 ml of DMEM without methionine and cysteine (DMEM⁻) for 1 h. Pulse-labelling (for 5 h at 37 °C) was carried out by the addition of 150 µCi of [³⁵S]methionine/cysteine (Pro-mix from Amersham Biosciences; specific activity < 1000 Ci/mmol) to the medium. After the pulse the medium was collected and the cells were lysed for α -amylase immunoprecipitation. BFA treatment involved addition of the drug (10 µg/ml final concentration) 1 h before the beginning of the pulse, and the maintenance of this level until the end of the experiment. When mentioned, tunicamycin (5 µg/ml final concentration) was added to the cell culture medium the day before the experiment and maintained at this level throughout all manipulations.

Samples were processed for immunoprecipitation as described below. The cell culture medium was cleared by centrifugation, and then radioimmunoprecipitation assay (RIPA) buffer [16] was added to it (one-half of the sample volume). Cells were solubilized in the cell culture dish by the addition of 500 µl of RIPA buffer, and the lysate was then transferred to a fresh tube. The dish was washed with 1 ml of PBS, and the wash was added to the lysate. Non-specific Protein A-binding proteins were removed from all samples by adding 30 µl of 10% (v/v) Protein A-Sepharose (Amersham Biosciences) and incubating for 1 h at 4 °C with stirring. After removal of the beads by centrifugation, immunoprecipitation was performed by adding 1 µl of anti-amylase and incubating overnight at 4 °C in the presence of 20 µg/ml PMSF, 0.5 µg/ml leupeptin and 100 µM EDTA. The immune complexes were harvested by adding 10 µl of Protein A-Sepharose and incubating for 1 h at 4 °C. After pelleting, the beads were washed twice with 50 mM Tris, 1% (v/v) Triton X-100 and 150 mM NaCl (pH 8.0), once with the same buffer containing 300 mM NaCl, and once with 50 mM Tris (pH 8.0), and were finally resuspended in SDS sample buffer. The immunoprecipitates were run on SDS/7.5% (w/v) polyacrylamide gels that were fixed and then soaked for 30 min in a fluorographic reagent (Amplify; Amersham Biosciences) prior to autoradiography of the dried gels.

Transient transfections of COS-1 cells with cDNAs of wild-type and mutant α -amylases were used to express the secreted proteins. Cells in 25-cm² culture flasks were pulse-labelled for 18 h with 225 μ Ci of [³⁵S]methionine/cysteine. The labelled amylase secreted into the pulse medium was processed for immunoprecipitation. Stably transfected MDCK cells were also used to confirm the results observed in the transient COS-1 transfections. MDCK cells stably expressing α -amylase were plated in 6-well culture dishes and grown to 100% confluence. They were pulse-labelled (for 2 h at 37 °C) with 150 μ Ci of [³⁵S]methionine/cysteine in 800 μ l of DMEM⁻. Amylase secreted into the pulse medium was processed for immunoprecipitation as described. The relative proportions of the different bands were estimated by scanning of the autoradiographs and subsequent quantification using UN-SCAN-IT software (Silk Scientific, Orem, UT, U.S.A.). Scans were performed in the linear range of exposition of the autoradiographs.

In vitro transcription, translation and translocation

The wild-type and truncated rat amylase genes, subcloned in pRc/CMV, were transcribed *in vitro* using the RiboMaxTM Large Scale RNA Production System-T7 (Promega, Madison, WI, U.S.A.) as recommended by the manufacturer. This *in vitro* synthesized RNA was then translated *in vitro* using a *Xenopus* oocyte extract generously provided by Dr K. I. J. Shennan (Department of Molecular and Cell Biology, Institute of Medical Sciences, Aberdeen, Scotland, U.K.) [17]. Microsomes containing translocated proteins were purified from the translation reaction mixture by diluting the translation/translocation reaction 10-fold with cold 10% (w/v) sucrose in PBS and Microfuging at 16000 *g* for 15 min at 4 °C. After careful removal of the bulk of the resulting supernatant, the loose pellet was washed with 500 μ l of the sucrose/PBS solution, and the microsomes were pelleted as described above. The entire supernatant was then carefully removed and the microsomal pellet was resuspended in 50 μ l of 20 mM Mes, 5 mM CaCl₂ and 1% (v/v) Triton X-100 (pH 6.8). The resulting solutions were incubated at 21 °C for 15 min so as to ensure complete microsome lysis, and 5 μ l aliquots were analysed by SDS/PAGE.

Determination of α -amylase specific activity

Transient transfections of COS-1 cells with cDNAs of wild-type and mutant α -amylases were used to express the secreted protein in high amounts, in order to be able to determine the specific activity of the glycosylated and non-glycosylated forms of the enzyme. Pulse-labelling of the cells was performed at approx. 58 h post-transfection in 25-cm² culture flasks for 16 h with 225 μ Ci of [³⁵S]methionine/cysteine using the conditions described above for AtT20 cells. Proteins secreted into the medium during the entire pulse period were collected. The 2.5 ml sample was affinity-purified on a 500 μ l β -cyclodextrin-agarose column in 20 mM Mes (pH 6.8) [18]. Elution of the column was performed with 8 mM β -cyclodextrin, and the active fractions were pooled for concentration and desalting using Centricon-30 (Millipore). All purification procedures were performed at 4 °C. Amylase activity was assayed at 37 °C in triplicate at pH 6.2 using 2-chloro-4-nitrophenol- α -maltotriose (Intersect Systems, Longview, WA, U.S.A.) as the substrate [19]. The specific activity of the purified samples was expressed per c.p.m. of radioactivity when amylase was estimated by scintillation counting, and per pixel of the scanned amylase bands when estimated with a PhosphorImager[®] (STORM 840; Molecular Dynamics). Amylase units are expressed in μ mol of substrate hydrolysed/min.

RESULTS

In order to assess whether or not rat pancreatic α -amylase could be glycosylated at one or both of its N-glycosylation sequons, AtT20 cells stably transfected with α -amylase cDNA were pulse-labelled and the resulting labelled α -amylase was immunoprecipitated from both the cell culture medium and the cells. Immunoprecipitation of the α -amylase in the cell culture medium showed the presence of a single form of glycosylated α -amylase (Figure 1). A glycosylated species of slightly lower molecular mass was barely detectable in the cells (Figure 1, lane 1, arrow). Addition of the potent N-glycosylation inhibitor tunicamycin confirmed that the upper band was the product of N-glycosylation, and, conversely, that the 55 kDa band was not glycosylated. The presence of only one additional band of molecular mass higher than the main band at 55000 kDa argues in favour of only a single site being glycosylated on α -amylase. Quantitative scanning of the autoradiograph indicated that the glycosylated species represented $1.95 \pm 0.20\%$ (mean $\pm$ S.D., $n = 3$) of the secreted protein. In order to test the hypothesis that the low rate of glycosylation was due to a limited accessibility of the newly synthesized protein to oligosaccharyl transferase (OST), pulse-labelling was performed in the presence of the fungal metabolite BFA. This treatment is known to block all secretion, and to induce the collapse of the Golgi complex into the ER [20]. It allows vesicle recycling between the ER and the ER-Golgi intermediate compartment, thereby putting all newly synthesized proteins into contact with all ER and Golgi enzymes involved in glycosylation. We believed that under these conditions any polypeptide that had escaped glycosylation would have ample opportunity to acquire carbohydrates, maximizing the amount of the glycosylated form present. Treatment with BFA did permit detection of the glycosylated form within the cell (Figure 1, BFA); however, the rate of glycosylation was no higher than that observed in secretions performed in the absence of BFA (Figure 1, lane 2). Clearly the BFA data show that the sequon accessible to glycosylation in the rat pancreatic α -amylase cannot be further glycosylated, even if its accessibility to OST is increased.

In order to determine whether Asn⁴¹⁰ or Asn⁴⁵⁹ was modified by OST, each residue was separately mutated to glutamine. We chose to substitute glutamine for asparagine so as to maintain both the charge and the polarity of the original side chain. In addition, we also created a clone in which both sequons were mutated. Since secretions were observed to contain the highest

Figure 1 Secretion of a monoglycosylated form of the rat pancreatic α -amylase from stably transfected AtT20 cells

Cells (C) and medium (M) of a 5 h pulse with [³⁵S]methionine/cysteine were immunoprecipitated with anti-amylase. A doublet is observed in secretions (lane 2). The upper band is produced by N-glycosylation of the protein as it is absent in the presence of the glycosylation inhibitor tunicamycin (Tnc). The presence of BFA does not increase the rate of glycosylation, but allows visualization in the cells of a glycosylated precursor migrating at a slightly lower molecular mass than the secreted mature form (lane 2). In untreated cells, this precursor is barely visible, and is indicated by an arrow (lane 1).

Figure 2 Glycosylation of rat pancreatic α -amylase mutants in COS-1 and MDCK cells

The two asparagine residues susceptible to N-glycosylation in the rat pancreatic α -amylase sequence at positions Asn⁴¹⁰ (N₁) and Asn⁴⁵⁹ (N₂) were alternatively mutated to glutamine (Q₁N₂ and N₁Q₂), or were simultaneously mutated (Q₁Q₂). The wild-type α -amylase (N₁N₂) was glycosylated in the same relative proportion as was observed in AtT20 cells (see Figure 1). Expression in transiently transfected COS-1 cells (left-hand panel) and in stably transfected MDCK cells (right-hand panel). The levels of protein expression differed between mutants, as well as between cell lines.

proportion of the glycosylated form (Figure 1), the three mutants were transiently transfected into COS-1 cells. Only the Asn⁴¹⁰ → Gln mutant showed a significant increase in the proportion of the glycosylated band, going from approx. 2% in the wild-type protein (N₁N₂ in Figure 2, left-hand panel) to $89.8 \pm 4.6\%$ in the mutant (Q₁N₂). The low rate of glycosylation of the wild-type protein in COS-1 cells was identical with that observed in AtT20 cells (Figure 1). The Asn⁴⁵⁹ → Gln mutation and the double mutant Asn⁴¹⁰ → Gln/Asn⁴⁵⁹ → Gln prevented glycosylation altogether. These observations confirmed that the protein was N-glycosylated at a single asparagine residue, and that the target for this modification was Asn⁴⁵⁹. Identical results were obtained when the mutants were stably expressed in the MDCK cell line (Figure 2, right-hand panel).

Since the site exposed to glycosylation was exclusively Asn⁴⁵⁹, and that the only anticipated hindrance to its glycosylation in the wild-type was its location in a disulphide loop, it was decided to

Figure 3 Glycosylation in COS-1 cells of the rat pancreatic α -amylase with a mutated disulphide loop in the C-domain

The two cysteine residues that form the disulphide loop in the C-domain of the rat pancreatic α -amylase were mutated to serine residues (Cys⁴⁴⁸ → Ser and Cys⁴⁶⁰ → Ser). When expressed in transiently transfected COS-1 cells, the secreted protein was quantitatively N-glycosylated. The glycoprotein was sensitive to release of the oligosaccharide by PNGase F (F), but not by Endo H (H). Because the level of protein expression differed between the mutant and the wild-type, the gel was exposed for two different periods of time in order to clearly compare the higher molecular mass and sensitivity to PNGase F of the mutant (top and middle panels), and to demonstrate the absence of any non-glycosylated mutated protein (bottom panel, —).

Table 1 Specific activities of wild-type and three glycosylation mutants of rat pancreatic α -amylase secreted by transiently transfected COS-1 cells

Specific activities were determined at 37 °C. Results are presented as means $\pm$ S.D.

Mutation	α -Amylase specific activity	
	μ mol of substrate hydrolysed/min per c.p.m.*	μ mol of substrate hydrolysed/min per pixel†
Wild-type	16 ± 3	657 ± 50
Asn ⁴¹⁰ → Gln	16 ± 2	589 ± 33
Asn ⁴⁵⁹ → Gln	16 ± 2	539 ± 10
Asn ⁴¹⁰ → Gln/Asn ⁴⁵⁹ → Gln	17 ± 3	543 ± 19

* C.p.m. were determined by scintillation counting of the affinity-purified samples.

† Pixels were quantified by PhosphorImager® analysis of the scanned α -amylase-labelled bands separated by SDS/PAGE.

destroy the loop by mutation of the two cysteine residues to Ser⁴⁴⁸ and Ser⁴⁶⁰. Upon transient expression of the mutant in COS-1 cells the protein was fully glycosylated (Figure 3), thus confirming our hypothesis that the disulphide loop is a major obstacle to glycosylation. The sensitivity to peptide N-glycosidase F (PNGase F), and not to endo- β -N-acetylglucosaminidase H (Endo H), showed that complex glycans were present on this glycosylated mutant amylase.

In order to determine the specific activities of the different α -amylase mutants so as to be able to compare them, the enzymes had to be purified from contaminating activities, and the amount of α -amylase polypeptide precisely determined. Unfortunately the amount of protein produced by the transfected cells was too small to use standard protein determination methods. We therefore developed a protocol to quantify the secreted α -amylase. The α -amylase was first affinity-purified on β -cyclodextrin-agarose, and the active fractions were pooled, concentrated and their α -amylase activity determined. The amount of α -amylase polypeptides was then determined by scanning SDS/PAGE gels using a PhosphorImager®. In fact, if the initial purification step was sufficiently efficient, then direct scintillation counting of the purified sample should yield comparable results. Accordingly, the radioactivity of all affinity-purified samples was estimated by scintillation counting. We found that the α -amylase specific activity of all mutants was not significantly different from that of the original protein (Table 1). The affinity-purified fractions were identical (results not shown) to the immunoprecipitated material shown in Figure 2, clearly explaining why the results obtained by gel scanning and liquid-scintillation counting produced similar relative specific activities. From these determinations it can be concluded that glycosylation has no effect on rat pancreatic α -amylase enzymic activity.

All these mutations in the C-domain did not influence the activity of the enzyme, suggesting that the C-domain is not essential for enzymic activity. This question was directly and drastically addressed by deleting the 65 C-terminal residues of the protein. Assuming that the structure of the rat enzyme is similar to the human homologue, this deletion would encompass the last seven β -strands of the C-domain, leaving three of the four β -strands that are in close proximity to the A domain. When this Δ C β 4–10 mutant was transiently expressed in COS-1 cells no amylase activity or any immunoreactive material was detected in the medium (Figure 4A). Instead, all of the protein was found to remain inside the cells. These observations indicated that the α -amylase C-domain is essential for the correct post-translational folding and secretion of the protein. In order to assess the

Figure 4 *In vitro* transcription, translation and translocation of the wild-type and C-terminal truncated rat pancreatic α -amylase

The 65 residues that form the last seven β -strands in the C-domain of rat pancreatic α -amylase were deleted by inserting a stop codon after the Asn⁴²⁹ codon. When expressed in transiently transfected COS-1 cells (Δ C β 4–10), the protein was retained in cells and was not secreted (A). After *in vitro* transcription, translation and translocation in *Xenopus* oocyte extracts, the wild-type protein produced a doublet and the truncated protein (Δ C β 4–10) produced a single band at 46 kDa (B).

potential activity of the truncated Δ C β 4–10 protein, the cDNA was transcribed, translated and translocated *in vitro*. A source of ER membranes different from the commonly used canine pancreatic membranes was required because of the high endogenous level of amylase in these preparations. *Xenopus* oocyte extracts are very efficient in translocation and post-translational modification [21], and they were used as a convenient source of ER membranes. As shown in Figure 4(B), the wild-type protein was efficiently translocated as a glycoprotein doublet. The truncated Δ C β 4–10 mutant was also efficiently translocated and displayed a single non-glycosylated band at the expected molecular mass of 46 kDa. No amylase activity was detected for Δ C β 4–10, whereas the wild-type was very active after translocation (results not shown). These observations revealed that the α -amylase C-domain must be present during translation and translocation to produce an active enzyme that can be secreted.

DISCUSSION

Even if three reports [3–5] identify pancreatic amylase purified from natural sources as a glycoprotein, it is at such a low level that the protein has always been considered as being non-glycosylated. This is somewhat surprising as both the rat and human enzymes possess two potential sites for N-glycosylation. These sites are located at equivalent positions in the two enzymes (Asn⁴¹⁰ and Asn⁴⁵⁹ in the rat, and Asn⁴¹² and Asn⁴⁶¹ in the human), and, in the structure of the human enzyme [2], are located on the surface of the α -amylase C-domain. In the present report, in which we describe the first expression of the rat pancreatic α -amylase in cultured heterologous cells, we directly address the question of animal amylase glycosylation.

The heterologous expression of wild-type rat amylase in AtT20 cells results in a very low rate of glycosylation (approx. 2%). Similar results were obtained when the gene was expressed in two other types of cells (MDCK and COS-1 cells), indicating that the low rate of glycosylation is not specific to a particular mammalian cell type. Our observation that only 2% of the rat α -amylase is glycosylated explains why many investigators have concluded that α -amylase purified from natural sources is non-glycosylated, as well as why a fraction of the enzyme could, at the same time, have some affinity for lectins [4]. When the human pancreatic α -amylase was expressed in yeast [14], the level of glycosylation of the protein was so significant that it interfered with crystallization. However, this behaviour could be a characteristic of yeast, which possesses totally different Golgi transferases [22], and is known to produce unpredictable glycosylation patterns

[23]. These studies in yeast with the human pancreatic α -amylase showed that Asn⁴⁶¹ (equivalent to rat Asn⁴⁵⁹) was the only target of glycosylation [14], and that no glycosylation was observed on Asn⁴¹², the upstream sequon in the human sequence (equivalent to rat Asn⁴¹⁰). Our results with the rat enzyme confirm these observations, specifically that the Asn⁴¹⁰ site is not glycosylated and that Asn⁴⁵⁹ is only slightly glycosylated. In addition, even if we artificially prolong the contact between the newly synthesized rat α -amylase and OST, by the addition of BFA, the proportion of the glycosylated protein does not increase. Clearly the structure of this region of the molecule does not show any sign of post-translational processing that is favoured during the BFA incubation and would therefore lead to an increased susceptibility to glycosylation. These experiments with BFA prompt the conclusion that the structural constraints on amylase with respect to its interaction with OST are already present at the initial presentation of the newly synthesized α -amylase to OST during translocation.

X-ray crystallography of human pancreatic α -amylase has shown that a strong hydrogen bond exists between Asn⁴¹² and Asp⁴³² (equivalent to Asn⁴¹⁰ and Asp⁴³⁰ in the rat) [14]. This interaction has been put forward to explain why Asn⁴¹² was not glycosylated at all when the protein was produced in yeast [14]. In theory, this sequon should be highly susceptible to glycosylation due to its position in an Asx turn at the surface of the protein [24]. However, it is difficult to understand that only Asp⁴³⁰ could cause such an impediment to glycosylation. A more probable explanation is that close-range interactions are involved in the repression. Foremost among the potential candidates are two hydrogen bonds formed between Asn⁴¹⁰ and Gln⁴¹⁴ on one side, and Asn⁴¹⁰ and Ser⁴¹² on the other. In fact, the Asn⁴¹⁰ OD1 ('oxygen 1 in the delta position') forms a strong hydrogen bond to the peptide nitrogen of Ser⁴¹² and thus indeed contributes to maintaining the overall structure in this region. Furthermore, the Asn⁴¹⁰ ND2 ('nitrogen 2 in the delta position') forms a hydrogen bond to Gln⁴¹⁴ OE1 ('oxygen 1 in the epsilon position'), clearly indicating that the most important factors reducing the accessibility of Asn⁴¹⁰ to OST in the rat pancreatic α -amylase are the hydrogen bonds with Gln⁴¹⁴ and Ser⁴¹². This theory is supported by our observation that the amylase mutant truncated at its C-terminus at Asp⁴²⁹ was not glycosylated.

The other potential glycosylation site in rat α -amylase is Asn⁴⁵⁹. As we show in the present study this residue is glycosylated at an extremely low rate in the wild-type protein, probably because it is located in the middle of a disulphide bridge between Cys⁴⁴⁸ and Cys⁴⁶⁰ that hinders glycosylation [25,26]. Due to the proximity of these two residues, this disulphide loop is probably already formed when Asn⁴⁵⁹ comes into contact with OST. The quantitative glycosylation of amylase when the disulphide bridge is disrupted by mutation of the cysteine residues clearly shows that this was the major structural hindrance to the glycosylation of Asn⁴⁵⁹.

The Asn⁴¹⁰ $\rightarrow$ Gln mutation was originally conceived as one conservative for both the charge and polarity of the side chain. The choice was made at the expense of the length of the side chain. By reference to the structure of the human homologue [2], lengthening of the lateral chain of Asn⁴¹⁰ could have two effects. It most likely impeded the establishment of the hydrogen bond between Asn⁴¹⁰ and Asp⁴³⁰ by putting the Gln⁴¹⁰ carbonyl in too close proximity to the Asp⁴³⁰ carboxy group for hydrogen-bond formation to occur. Secondly, the mutation could have brought the side chain of Gln⁴¹⁰ very close to Trp⁴³², thus creating an interaction whose effect, while impossible to predict, quite likely gave rise to a long-range conformational perturbation down the polypeptide backbone. It can therefore be concluded that the

hydrogen bonds between Asn⁴¹⁰ and both Ser⁴¹² and Gln⁴¹⁴, as well as the one between Asn⁴¹⁰ and Asp⁴³⁰, are major determinants in the preservation of the original structure of this α -amylase subdomain. Additionally, these interactions may play a role in the formation of the disulphide loop between Cys⁴⁴⁸ and Cys⁴⁶⁰.

In order to address the structure–function relationship of α -amylase glycosylation, the specific activity of the enzyme had to be determined. Unfortunately the amounts of protein expressed in cultured cells are too low to be biochemically determined. We resolved this problem using two approaches: quantification of radioactive bands on scanned gels, and scintillation counting of the affinity-purified enzyme. The two methods yielded equivalent relative specific activities, thereby establishing the reliability of the technique. Most importantly, these experiments revealed that all the α -amylases expressed, mutants as well as wild-type, had the same specific activity regardless of their level of glycosylation. Clearly, neither glycosylation nor the disulphide loop of the C-domain is involved in the enzymic activity of α -amylase.

While the mutations had severe enough effects on the C-domain structure to induce or completely inhibit glycosylation at Asn⁴⁵⁹, none of them affected the enzyme activity harboured in the A- and B-domains. In addition, none of the mutations altered protein folding to the point of inducing intracellular retention or degradation. They confirmed the relative structural independence of the C-domain with respect to the N-terminal region of the protein [11]. However, deletion of 70% of the C-domain sufficiently impeded folding of the protein so as to make its secretion impossible from transfected cells. From these observations it appears that this domain is essential for both the biosynthesis and the secretion of amylase, but that it can accommodate many structural variations, in a manner reminiscent of the diversity of such structures seen in glycohydrolases.

In summary, using an approach of mutation and deletion we have produced new information on the essential role of the C-domain of pancreatic α -amylase for the post-translational maturation and folding of the protein. In addition, we have identified Asn⁴¹⁰ as a key residue that is required for maintaining the integrity of the C-domain and its susceptibility to N-glycosylation.

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