The EMBO Journal vol.15 no.14 pp.3538–3546, 1996

# Competition between folding and glycosylation in the endoplasmic reticulum

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**Using carboxypeptidase Y in *Saccharomyces cerevisiae* as a model system, the *in vivo* relationship between protein folding and *N*-glycosylation was studied. Seven new sites for *N*-glycosylation were introduced at positions buried in the folded protein structure. The level of glycosylation of such new acceptor sites was analysed by pulse-labelling under two sets of conditions that are known to reduce the rate of folding: (i) addition of dithiothreitol to the growth medium and (ii) introduction of deletions in the propeptide. A variety of effects was observed, depending on the position of the new acceptor sites. In some cases, all the newly synthesized mutant protein was modified at the novel site while in others no modification took place. In the most interesting category of mutants, the level of glycosylation was dependent on the conditions for folding. This shows that folding and glycosylation reactions can compete *in vivo* and that glycosylation does not necessarily precede folding. The approach described may be generally applicable for the analysis of protein folding *in vivo*.**

*Keywords*: carboxypeptidase Y/disulphide bonds/protein engineering/protein structure/yeast

## Introduction

The modification of Asn–Xaa–Ser and Asn–Xaa–Thr sequences with oligosaccharides is one of the ubiquitous features of the eukaryotic secretory pathway. This process, which is catalysed by a complex enzymatic machinery in the endoplasmic reticulum (ER) (Kelleher *et al.*, 1992; Kelleher and Gilmore, 1994; Knauer and Lehle, 1994; Kumar *et al.*, 1995), takes place in conjunction with folding of the newly translocated polypeptide. Several lines of evidence suggest that there is an intimate interplay between the folding and glycosylation processes. It is, for instance, known that absence of glycosylation at certain sites may reduce the rate of folding of some secretory glycoproteins *in vivo* (Machamer *et al.*, 1985; Taylor and Wall, 1988; Reiderer and Hinnen, 1991; Winther *et al.*, 1991), suggesting that glycosylation occurs before the final steps in folding. On the other hand, a number of questions relating to the steric and kinetic interplay between the newly translated/translocated glycoprotein and the glycosyl transferase remain unanswered. For instance, it is not known why all potential glycosylation sites are not utilized (Gavel and von Heijne, 1990), even though *in vitro* analysis suggests that there is no significant sequence specificity of the glycosyl transferase, apart from the requirement for the absence of Pro in the central position of the glycosylation sequon Asn–Xaa–Ser/Thr (Bause, 1983; Lehle and Bause, 1984; Imperiali and Shannon, 1991). Recent work, however, suggests that there may be a link between folding and the utilization of glycosylation sequons *in vivo* (Allen *et al.*, 1995). This work shows that inhibition of disulphide bond formation may increase the level of modification of a naturally occurring glycosylation sequon in tissue-type plasminogen activator.

Folding of the polypeptide chain inside the cells can, in principle, begin as soon as the N-terminal part of the nascent chain emerges from the ribosome, be it in the cytosol or via channels into, for example, the ER (Bergman and Kuehl, 1979; Braakman *et al.*, 1991; Chen *et al.*, 1995; Fedorov and Baldwin, 1995). Folding of newly synthesized polypeptides probably begins with the formation of local secondary structures (Hlodan and Hartl, 1994). The subsequent steps have not been analysed in detail as few techniques have been available.

The secretory pathway and the *N*-glycosylation apparatus are well characterized in *Saccharomyces cerevisiae* (Pryer *et al.*, 1992; Kelleher and Gilmore, 1994; Knauer and Lehle, 1994), and *N*-linked glycosylation requires the sequon conserved in other eukaryotes. Also in yeast, not all sequons are utilized. For example, vacuolar proteinase B contains five glycosylation sequons, but only one of them is utilized (Moehle *et al.*, 1987). If the N-terminal propeptide of proteinase B is deleted, however, one additional sequon becomes glycosylated (Nebes and Jones, 1991). This observation suggests that structure of higher order than local secondary structure can influence the level of glycosylation at a given sequon. In order to investigate the relationship between folding and glycosylation in the ER, we have chosen to investigate the well-characterized secretory protein, carboxypeptidase Y (CPY), for which the three-dimensional structure is known (Endrizzi *et al.*, 1994). CPY is, furthermore, a very convenient model protein since it can be followed easily through the secretory pathway by pulse-labelling and immunoprecipitation. Thus, CPY is synthesized with a cleavable pre-sequence that targets the precursor to the ER. Inside the ER, the wild-type proCPY becomes glycosylated at four acceptor sites for *N*-linked glycosylation, giving rise to a characteristic p1-form (67 kDa). Furthermore, folding of the proenzyme takes place concurrently with the formation of five disulphide bonds. After folding into a transport-competent form, proCPY continues to the Golgi apparatus, where the glycosyl side chains are extended to give the 69 kDa p2-form. Finally, upon reaching the vacuole, the propeptide is cleaved off, leading to the mature CPY form (61 kDa). Refolding

studies of CPY *in vitro* (Winther and Sørensen, 1991; Winther *et al.*, 1994) have shown that the propeptide plays a key role in attaining the correctly folded structure. In addition, *in vivo* analysis of various propeptide-deleted forms of CPY has revealed a slow rate of ER exit, indicative of a slow rate of folding in that compartment (Ramos *et al.*, 1994).

The present investigation is based on the introduction of new *N*-glycosylation sequons in regions which were predicted to be rendered rapidly inaccessible by collapse with more amino-proximal parts of the protein. The presence or absence of glycosylation at the new sequons would then give information on the kinetics of early folding events relative to glycosylation. Seven novel sequons were introduced into CPY. Some of the novel sites were not glycosylated in otherwise normal CPY under normal growth conditions. However, reduction of folding efficiency, by inhibition of disulphide bond formation or deletion of part of the propeptide, leads to enhanced utilization of some of these sites. To our knowledge, this is the first demonstration, in a well-defined model system, of a kinetic interrelationship between folding and *N*-glycosyl transfer reactions. We have also found that Asn–Xaa–Thr sequons at identical sites are better substrates *in vivo* for the glycosyltransferase than are Asn–Xaa–Ser sequons. The results suggest that introduction of new sites for *N*-linked glycosylation may constitute a sensitive assay for analysis of folding of secretory proteins *in vivo*.

## Results

### *Experimental approach*

We were interested in selecting new glycosylation sites in CPY, hoping to find some at which utilization was dependent on the overall rate of folding of the enzyme. In deciding where to introduce such sites, we tried to adhere to the following four criteria. (i) Since the formation of secondary structure (e.g. α-helices) is likely to be one of the earliest folding events (Matthews, 1993; Dobson *et al.*, 1994), we sought out such structures as prime targets. (ii) We attempted to introduce the new sites at positions predicted to be inaccessible, as deduced from the crystal structure of CPY (Endrizzi *et al.*, 1994). (iii) We tried to introduce sites at positions where the amino acid sequence was not conserved between various homologous serine carboxypeptidases (Olesen and Breddam, 1995). This was to avoid the possibility that the mutations *per se* would disrupt the ability of the protein to form a native structure, primarily because presentation of the results would be more complicated. The goal was to identify positions where the level of glycosylation of a particular site is dependent on the overall folding efficiency of the protein. Since we were interested in monitoring the formation of higher order structures, it is of less concern whether a mutation affects the formation of local structures. (iv) Since proline residues are probably not compatible with glycosyl transfer, such residues were avoided in the middle of the sequon. We constructed seven mutant forms of CPY (CPY-1S through to CPY-7S) by changing existing sequences to Asn–Xaa–Ser sequons (Table I). Their locations in the tertiary structure of CPY are indicated in Figure 1. Serine was chosen initially at the third sequon position since it was expected that this residue might reduce the affinity of the glycosyl transferase, and thereby would be more likely to reveal sites where folding was rapid. The nomenclature adopted identifies the new sequon by a number (1–7 starting from the most N-proximal site) and the identity of the residue at the third position in each sequon (e.g. CPY-3S for Asn–Xaa–Ser at sequon number 3).

To determine the level of glycosylation at the new sequons, mutants were analysed by $^{35}$S-pulse-labelling and immunoprecipitation of CPY antigen, followed by separation on SDS–PAGE. Glycosylation at a novel site increases the molecular weight by ~1.5 kDa. As described in the Introduction, CPY undergoes a number of modifications during transit through the secretory pathway. The pulse-labelling analysis not only reveals whether or not the new sequon is glycosylated, but the later processing (Golgi modification and proteolytic maturation) also gives information on the rate of intracellular transport and stability of the mutant form. Using this basic tool, various types of experiments were carried out. (i) To examine the direct effects of the amino acid exchanges, pulse-labelling was performed in the presence of tunicamycin, which inhibits *N*-glycosylation. In the absence of *N*-glycosylation, wild-type and mutant CPY should be folded and exit the ER at the same rate if the mutations *per se* did not affect folding. (ii) Addition of an extra core glycosyl unit gives reduced mobility on SDS–PAGE roughly equivalent to that resulting from mannosyl modifications taking place in the Golgi compartment. Thus, a *sec18* strain was used in some experiments to prevent the precursors from reaching this organelle (Graham and Emr, 1991). This allows for unequivocal determination of the level of overglycosylation. (iii) It was particularly interesting to see if there were sites that were not glycosylated under normal growth conditions but were glycosylated when folding was impaired. In order to reduce folding efficiency *in vivo*, we firstly investigated some of the mutants in the presence of dithiothreitol (DTT). DTT has been shown to affect the formation of disulphide bridges in the nascent chain *in vivo*, both in mammalian cells and yeast (Braakman *et al.*, 1991; Jämsä *et al.*, 1994; Simons *et al.*, 1995). Secondly, we combined the glycosylation sequon mutants with deletions in the propeptide, since there is strong evidence that folding is compromised by such deletions (Ramos *et al.*, 1994). (iv) Finally, we wished to consider the possibility that the utilization of certain sites under conditions perceived as being unfavourable to folding were just a consequence of retention in the ER for extended periods of time. For this purpose, we employed a *sec23* strain which is blocked in exit from the ER (Ferro-Novick *et al.*, 1984; Graham and Emr, 1991). Using these tools, a wide range of phenotypes were observed and in the following we shall describe some of them.

### *Characterization of mutant carboxypeptidase Y forms with new Asn–Xaa–Ser N-glycosylation sites*

Figure 2 shows an SDS–PAGE autoradiogram of $^{35}$S-labelled CPY antigen from cells producing wild-type CPY or mutant CPY-1S. Addition of non-radioactive methionine stops the labelling and the wild-type precursors are matured with a half-time of 5–7 min (Figure 2, lanes 1–3). After

**Table I.** Plasmids used in this study

| Name[a] | *PRC1* allele | Enzyme[b] | Amino acid changes[c] | Oligo[d] |
|---|---|---|---|---|
| pWI-3[e] | *PRC1* | | | |
| pJW1433[f] | *PRC1* | CPY | | |
| pCRR-312[f] | *prc1-Δ47/71* | Δ47/71CPY | | |
| pYSP1[g] | *PRC1* | | | |
| pWI-17[h] | *prc1-Δbam* | | | |
| pBH566[h] | *prc1-Δ47/71* | | | |
| pBH575[h] | *prc1-Δ47/71-Δbam* | | | |
| pBH584[h] | *prc1-Δ47/71-Δbam* | | | |
| pBH605[h] | *prc1-605* | Δ47/71CPY-4S | I386N, D388S | oBH18 |
| pBH609[h] | *prc1-609* | CPY-1S | I332N | oBH15 |
| pBH616[h] | *prc1-616* | CPY-5S | N416S | oBH19 |
| pBH623[h] | *prc1-623* | CPY-2S | A354S | oBH16 |
| pBH629[h] | *prc1-629* | CPY-7S | L434N, N436S | oBH21 |
| pBH637[h] | *prc1-637* | CPY-4S | I386N, D388S | oBH18 |
| pBH639[h] | *prc1-639* | CPY-6T | A430N | oBH20 |
| pBH800[h] | *prc1-800* | Δ47/71CPY-5S | N416S | oBH19 |
| pBH845[h] | *prc1-845* | Δ47/71CPY-6T | A430N | oBH20 |
| pBH923[h] | *prc1-923* | CPY-3S | Q355N, A357S | oBH17 |
| pBH929[h] | *prc1-929* | CPY-4T | I386N, D388T | oBH33 |
| pBH933[h] | *prc1-933* | CPY-5T | N416T | oBH34 |
| pBH936[h] | *prc1-936* | Δ47/71CPY-3S | Q355N, A357S | oBH17 |
| pBH941[h] | *prc1-941* | Δ47/71CPY-4T | I386N, D388T | oBH33 |
| pBH945[h] | *prc1-945* | Δ47/71CPY-5T | N416T | oBH34 |
| pBH991[h] | *prc1-991* | CPY-6S | A430N, T432S | oBH35 |
| pBH994[h] | *prc1-994* | CPY-3T | Q355N, A357T | oBH38 |
| pBH1011[h] | *prc1-1011* | Δ47/71CPY-3T | Q355N, A357T | oBH38 |
| pBH1177[h] | *prc1-1177* | Δ47/71CPY-6S | A430N, T432S | oBH35 |

[a]All plasmids contain the *CEN*, *ARS1* and *URA3* yeast functions, except pWI-3 and pYSP1, which have the 2 μ, and pWI-17, which has no sequences for maintenance in yeast.
[b]Nomenclature: *Δbam* indicates that a *Bam*HI–*Bam*HI fragment, containing most of the *PRC1* gene, has been deleted. Δ47/71 indicates that amino acids 47–71 have been deleted. Numbers 1–7 indicate the introduced acceptor site at positions 332, 352, 355, 386, 414, 430 and 434, respectively. Codon number 1 is defined as the ATG start codon of the *PRC1* open reading frame (Valls *et al.*, 1987). S and T indicate a Ser or Thr, respectively, at the third position of the glycosylation sequon.
[c]Amino acid changes introduced by site-directed mutagenesis.
[d]Oligos used for introduction of the mutations (for sequences see Table III).
[e]Winther *et al.* (1991).
[f]Ramos *et al.* (1994).
[g]Olesen and Kielland-Brandt (1993).
[h]This work.

15 min of chase, almost all CPY antigen is in the mature form. The lower mobility of the p1-form of CPY-1S suggests that the new sequon in CPY-1S has a glycosyl chain attached (Figure 2, lane 4). The CPY-1S precursors are not matured, which strongly suggests misfolding and ER retention (Figure 2, lanes 4–6). In the presence of tunicamycin, CPY-1S is also retained in the pro-form and degraded with a half-life of ~60 min (data not shown), suggesting that the amino acid exchange in CPY-1S *per se* prevents proper folding.

Pulse-labelling showed that the CPY-2S mutant form also had an extra glycosyl chain attached. However, in this mutant, the maturation takes place at a normal rate (Figure 3). This suggests that the glycosyl side chain can be accommodated even though the asparagine side chain only appears to be slightly exposed to solvent (Figures 1 and 6).

The CPY-3S site is located in the C-terminal part of two successive α-helices. The side chain of Gln355 in the wild-type enzyme faces the core of the protein (Figure 1) and we expect that this will also be the case for the Asn residue which is introduced at this position in the 3S mutant. In pulse-labelling no glycosylation of the 3S sequon was seen and the rate of maturation was normal (Figure 4A, lanes 4–6). Similar analysis of the CPY-6S mutant showed that ~50% of the precursor molecules were modified at the 6S site (Figure 5, lanes 4–6). Interestingly, both the normally and overglycosylated forms of CPY-6S were matured at a normal rate.

Based on pulse-labelling experiments in $Sec^+$ and *sec18* strains, we found that in CPY-4S ~25% of the 4S sequons were glycosylated (Table II). The proCPY-4S containing this extra glycosyl chain was unstable and disappeared upon chase, whereas the normally glycosylated precursor was matured. Similar analysis of CPY-5S showed that no extra glycosyl residue was attached to the new sequon (Table II). However, no p2-form was seen and ~20% of the labelled protein was still present in the p1-form after 15 min of chase. Thus, while the introduction of a new *N*-glycosylation site at this position does not lead to any extra glycosylation, a reduced rate of maturation is observed. In the most C-proximal mutant, CPY-7S, only 10% of the precursor molecules have an extra glycosyl chain attached (Table II). No maturation could be detected in pulse-labelling experiments, and cells almost completely lacked CPY activity (data not shown). Thus, the introduced amino acid changes were probably incompatible with correct folding of the protein.

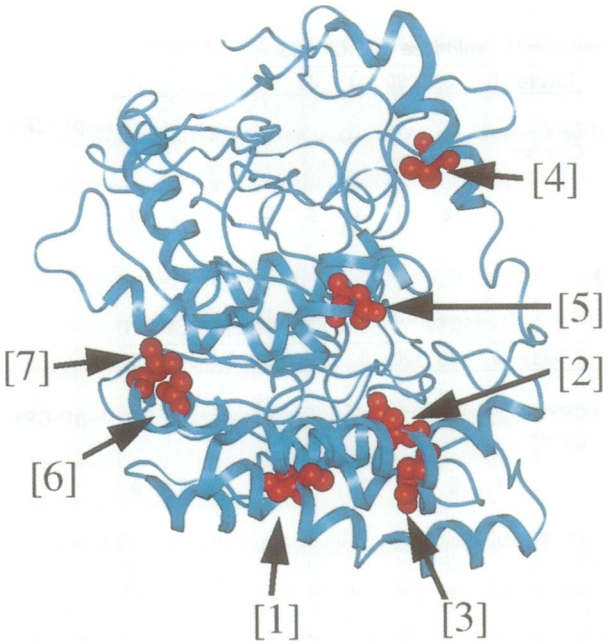



**Fig. 1.** Positions of introduced glycosylation sequons in carboxypeptidase Y. Wild-type residues (I332, N352, Q355, I386, N414, A430, L434) corresponding to the new glycosylation sites 1–7 are shown by ball and stick structures in red. The peptide backbone according to Endrizzi *et al.* (1994) is indicated with the blue ribbon structure.

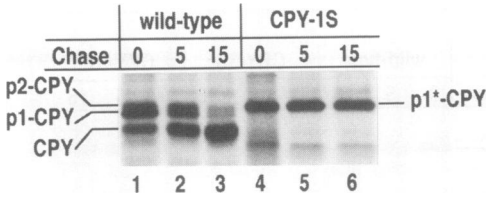



**Fig. 2.** Mutant CPY-1S has an extra glycosyl chain attached and is not matured. Cells were starved for methionine, pulsed with $^{35}$S-labelled methionine for 15 min and chased with unlabelled methionine for the indicated periods of time. Immunoprecipitation was followed by separation on SDS–PAGE. Lanes 1–3, CPY; lanes 4–6, mutant CPY-1S (new sequon Asn332–Glu–Ser). In wild-type cells, the band with the lowest mobility corresponds to the Golgi form (p2-CPY) of CPY, the middle band to the ER form (p1-CPY), whereas the fastest moving band represents the mature form of CPY (CPY). In mutant CPY-1S only one band is seen. The p1-form (p1*-CPY) of CPY-1S (lane 4) runs slightly more slowly than the p2-form of CPY, because of the extra glycosyl chain. The asterisk indicates overglycosylation.

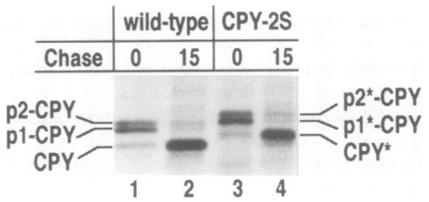



**Fig. 3.** Glycosylation of the introduced site in CPY-2S does not affect folding or maturation. Pulse-labelling and immunoprecipitation of CPY and CPY-2S were performed as described in Figure 2. Lanes 1 and 2, CPY; lanes 3 and 4, CPY-2S. In CPY-2S both precursors and the mature form are overglycosylated.

### *Asn–Xaa–Thr sites are glycosylated more efficiently than Asn–Xaa–Ser sites*

It has been shown that replacing Asn–Xaa–Ser sites by Asn–Xaa–Thr sites results in a 10-fold decrease in $K_m$ for the glycosyl transfer to hexapeptides *in vitro* (Bause and Legler, 1981). To investigate if Asn–Xaa–Thr sites in our mutants are also utilized to a greater extent *in vivo*, we replaced the Asn–Xaa–Ser site in mutants CPY-3S, -4S, -5S and -6S, which showed no or only partial glycosylation, with Asn–Xaa–Thr sites. The resulting four mutants, CPY-3T, -4T, -5T and -6T, subsequently were analysed in pulse-labelling experiments.

In mutants CPY-3T and CPY-5T no extra glycosylation was found (Figure 4A, lanes 7–9; Table II). However, in mutant CPY-4T ~70% of the newly synthesized proCPY-4T contained an extra glycosyl chain, compared with ~25% in CPY-4S (Table II). These overglycosylated precursors were unstable. Similarly, while 50% of the CPY-6S precursor molecules received an extra glycosyl chain, all CPY-6T was glycosylated (Figure 5, lanes 7–9). The extra glycosyl chain did not, however, affect the rate of maturation. These data are a clear *in vivo* confirmation that sequons containing Thr in the third position are better substrates for the glycosyl transferase.

### *The level of glycosylation can be affected by changing the conditions for folding*

Several groups have demonstrated that co-translational disulphide bond formation can be reversibly inhibited *in vivo* by the addition of DTT to the culture medium (Braakman *et al.*, 1991; Jämsä *et al.*, 1994; Simons *et al.*, 1995). Treatment of cells with DTT kept the newly synthesized proteins in a reduced form, but had no major effect on translation, translocation or *N*-glycosylation. They found, furthermore, that interference with disulphide bond formation affected folding, leading to retention in the ER. Subsequent removal of the DTT will allow these proteins to fold and leave the ER. CPY, which contains five disulphide bridges, follows this regimen (Simons *et al.*, 1995). In the following, we will concentrate on the CPY-3S, 3T and 6S mutants, which under normal growth conditions are not or are only partially glycosylated at the 3T/S and 6S positions. We posed the question whether these sites are more extensively glycosylated if folding is compromised by DTT treatment.

In the case of the CPY-3T mutant, the addition of DTT results in glycosylation of ~40% of the newly synthesized precursors. Chase with non-radioactive sulphur in the presence of DTT does not lead to further glycosylation (data not shown). However, if DTT is washed out prior to initiation of the chase, the precursor molecules with an extra glycosyl chain are degraded, whereas the normally glycosylated precursors are matured (Figure 4B, lanes 7–9). The glycosylation of the 3T site in the presence of DTT suggests that a lower efficiency of folding allows glycosylation in some cases. However, this could be an indirect effect, caused by the retention in the ER and not a direct result of the folding defect associated with the DTT treatment. To test this, we produced the CPY-3T mutant form in a *sec23* mutant, which, at the non-permissive temperature, is deficient in budding of vesicles from the ER membrane. Transport out of the ER was blocked for up to 60 min but this did not result in glycosylation at the 3T site (data not shown). Compared with the site 3T mutant, treatment of the CPY-6S mutant with DTT had a similar albeit less dramatic effect,

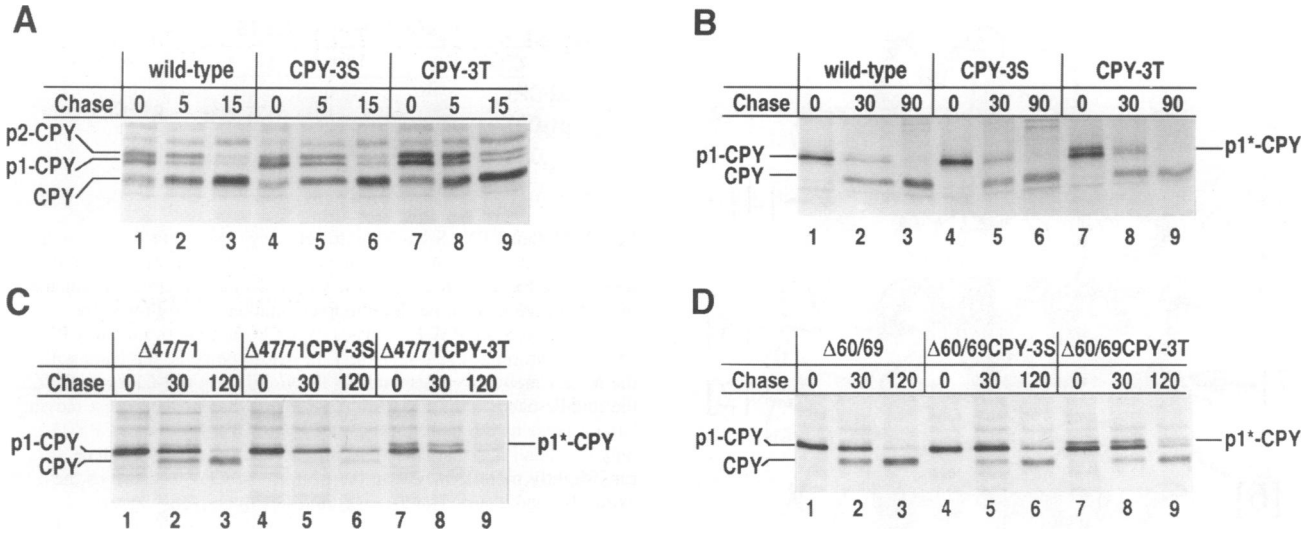



**Fig. 4.** Disfavouring folding leads to glycosylation of the new sequon in mutant CPY-3T. Pulse-labelling and immunoprecipitation of CPY and CPY-3 mutant forms were performed as described in Figure 2. (**A**) Lanes 1–3, CPY; lanes 4–6, CPY-3S (new sequon Asn355–Leu–Ser); lanes 7–9, CPY-3T (new sequon Asn355–Leu–Thr). The glycosylation level and maturation rate are not changed significantly in CPY-3S and CPY-3T. (**B**) Labelling in the presence of 20 mM DTT, chase without DTT; lanes 1–3, CPY; lanes 4–6, CPY-3S; lanes 7–9, CPY-3T. In CPY-3T, two ER forms appear, one with four and one with five glycosyl chains. This is indeed an ER form since it is already present at the first time point and since DTT has been shown to block ER exit of CPY (Simons *et al.*, 1995). (**C**) Combination with the *prc1*-Δ47/71 propeptide deletion. Lanes 1–3, Δ47/71CPY; lanes 4–6, Δ47/71CPY3S; lanes 7–9, Δ47/71CPY-3T. Due to the slow folding rate, the steady-state levels of the p2-form are too low to be seen. Consequently, the two forms seen in Δ47/71CPY3T are due to overglycosylation. (**D**) Combination with the *prc1*-Δ60/69 propeptide deletion. Lanes 1–3, Δ60/69CPY; lanes 4–6, Δ60/69CPY3S; lanes 7–9, Δ60/69CPY-3T.

increasing the level of glycosylation of this sequon in the newly synthesized protein from 50 to 70% (Table II). We also tested the level of glycosylation at the 3S site and found no effect of treatment of the cells with DTT. This observation shows that the glycosylation efficiency can be modulated by the choice of either serine or threonine residues at the third position of a given sequon. It also supports the general notion that folding and glycosylation at appropriate sites are in kinetic competition.

It has been shown previously that deletions in the CPY propeptide lead to a slower exit from the ER (Ramos *et al.*, 1994). There is strong *in vitro* evidence that this is due to inefficient folding of the propeptide-deleted forms. As an alternative approach to influence folding, we therefore chose to investigate a small propeptide deletion, Δ47/71, which has a half-time of ER exit of ~75 min (Ramos *et al.*, 1994). This propeptide deletion was combined with the 3T and 6S mutations. While no glycosylation takes place at the 3T position with a wild-type propeptide, ~40% of the Δ47/71-CPY-3T precursor molecules have a glycosyl chain attached to this position (Figure 4C, lanes 7–9). Similarly, the level of glycosylation at the 6S site increases from 50 to 70% when combined with the propeptide deletion (Table II). If the extra glycosylation is caused by less efficient folding, it should also be seen with other propeptide deletions that interfere with folding. To test this, the 3T sequon was combined with a propeptide deletion covering amino acid residues 60–69. This combination also exhibited the extra glycosylation (Figure 4D). Thus, blocking disulphide bond formation and deletions in the propeptide yield a similar effect. It is interesting that a combination of propeptide deletion and DTT treatment did not augment the level of glycosylation at any of the sequons analysed (Table II). In conclusion, the results show that the level of glycosylation of sequons buried in the protein structure can be modified by factors affecting the protein folding.

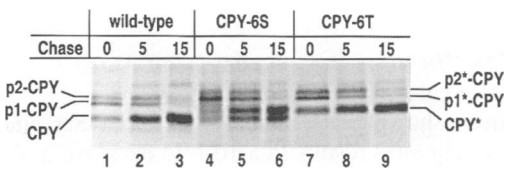



**Fig. 5.** Glycosylation of site 6 does not affect folding or maturation. Pulse-labelling and immunoprecipitation of CPY antigen were performed as described in Figure 2. Lanes 1–3, CPY; lanes 4–6, CPY-6S; lanes 7–9, CPY-6T. Around 50% of the CPY-6S molecules and all CPY-6T have an extra glycosyl chain attached.

The CPY-4S and 4T mutants show the same trend; however, these mutants appear to be structurally compromised by the increased level of glycosylation.

## Discussion

In the present work we have investigated the relationship between glycosylation and folding in the ER. It is well established that *N*-glycosylation in specific cases can promote folding and secretory transport, suggesting that part of the folding process can be preceded by glycosylation. The question of to what extent some folding steps can take place before glycosylation has been studied less extensively. We have generated seven new sequons for *N*-glycosylation in CPY at positions that would be expected to be inaccessible in the folded structure, and investigated the level of glycosylation of these mutants under different conditions for folding. In three of the seven new glycosylation sites that we have introduced, we find that the level of glycosylation is dependent upon the folding conditions.

A conspicuous feature of the CPY structure is a domain

**Table II.** Glycosylation levels of the new N–X–S/T sequons (%)

| Protein | DTT absent | DTT present |
|---|---|---|
| CPY | NA | NA |
| Δ47/71 CPY | NA | NA |
| CPY-1S | 100 | ND |
| CPY-2S | 100 | ND |
| CPY-3S | 0 | 0 |
| CPY-3T | 0 | 40 |
| Δ47/71 CPY-3S | 0 | 0 |
| Δ47/71 CPY-3T | 40 | 40 |
| CPY-4S | 25 | 40 |
| CPY-4T | 70 | 100 |
| Δ47/71 CPY-4S | 70 | 70 |
| Δ47/71 CPY-4T | 100 | 100 |
| CPY-5S | 0 | 0 |
| CPY-5T | 0 | 0 |
| Δ47/71 CPY-5S | 0 | 0 |
| Δ47/71 CPY-5T | 0 | 0 |
| CPY-6S | 50 | 70 |
| CPY-6T | 100 | 100 |
| Δ47/71 CPY-6S | 70 | 70 |
| Δ47/71 CPY-6T | 100 | 100 |
| CPY-7S | 10 | ND |

NA, not applicable; ND, not determined.

containing two α-helices that are held together by two disulphide bridges (Figure 6). One of the helices is further tied to the rest of the molecule by an additional disulphide bond. This structure thus engages three of the five disulphide bridges of the protein and has been termed a 'disulphide zipper' (Endrizzi *et al.*, 1994). The most informative of our mutants, CPY-3, has a new sequon at a position which is located in the C-proximal end of the second α-helix structure (Figure 6). Residue 355, which is subject to glycosylation in the mutant, protrudes into the body of the protein and is completely covered by the disulphide zipper structure (Endrizzi *et al.*, 1994; Figure 6). In pulse-labelling and immunoprecipitation experiments under normal growth conditions, we observe a normal rate of secretory transport (suggestive of a normal rate of folding) and find no glycosylation, irrespective of whether serine or threonine is in the third position of the sequon. However, when the formation of disulphide bonds is blocked by adding DTT to the cells, ~40% of the precursor molecules are glycosylated at the 3T site. A similar level of glycosylation is obtained when a deletion in the propeptide is combined with the 3T mutation.

What are the possible explanations for these results? In view of the prominence of the disulphide bonds in this part of the CPY structure we find it likely that maintaining them in the reduced state would be highly deleterious to the formation of a compact structure. One could argue that DTT may have a general effect which, by some unknown mechanism, would enhance the efficiency of the oligosaccharyl transferase. We therefore made use of propeptide deletions as an alternative way to impede folding. The fact that DTT treatment and deletion in the propeptide segment result in the same level of over-glycosylation argues strongly in favour of a change in folding pathway/kinetics being the main reason for the extra glycosylation. We find it likely that the presence of the intact propeptide inhibits glycosylation by generally promoting folding. Alternatively, it is a formal possibility that it creates a direct steric hindrance to glycosylation.

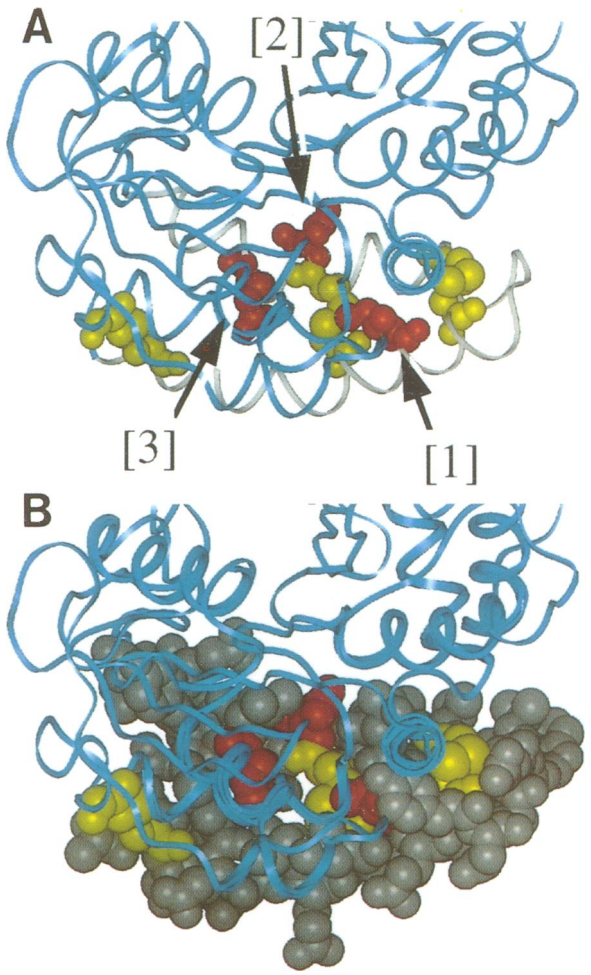
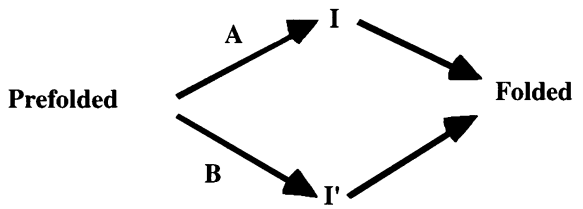



**Fig. 6.** Space-filling model of the two α-helices containing sites 1, 2 and 3. Wild-type residues (I332, N352, Q355) corresponding to the new glycosylation sites 1, 2 and 3 are shown in red. The relevant α-helicis are shown in grey. (**A**) The three glycosylation sites and the cystines involved in disulphide bridges (light green) in this domain are shown in a space-filling model. (**B**) Colour code as in (A) but with the two α-helices in a space-filling model (grey). The structural prominence of the disulphides in this part of the protein as well as the buried nature of sites 1 and 3 are apparent. The domain containing the two α-helices is seen as it appears from the core of the protein. Comparison with Figure 1 shows site 2 to be exposed to solvent.

In both cases, however, some steps of folding have preceded glycosylation. Several mutants (3T, 4S, 4T and 6S; Table II) show a similar glycosylation response to DTT treatment and propeptide deletion. This argues against a specific steric effect of the propeptide deletion. We have seen, furthermore, that another propeptide deletion extending from amino acid residue 60 to 69 has the same effect in combination with the 3T mutation (Figure 4D). The fact that combining the 3T mutation with the Δ47/71 propeptide deletion results in degradation can be said to represent synergism. This, however, only underscores the conclusion that the increased level of glycosylation is a result of reduced folding efficiency. In principle, it is possible, furthermore, that the glycosylation of an introduced site under normal folding conditions impairs the glycosylation of an endogenous (i.e. wild-type) downstream site. We find this highly unlikely for the following reasons. It can be seen from the three-dimensional structure

of CPY that the 3T site is for all practical purposes inaccessible to solvent [calculations using the NACCESS program by Hubbard and Thornton (1993) show that only 0.1% of the original Gln355 residue is water-accessible]. Glycosylation would most certainly destroy the ability of the protein to fold. That this is indeed the case is apparent from Figure 4D where none of the CPY which is glycosylated at this position (p1*-CPY) is converted to the mature form. We have purified the mature protein CPY-3T and found it to be stable and active (data not shown). This would be highly unlikely if the 3T site was glycosylated. Finally, the only site downstream of the 3T site (Asn479) is localized on the opposite side of the protein with respect to the 3T site, excluding mutual interaction. It is important to note that even if an endogeneous glycosylation site were not used under normal folding conditions, the increased use of this site under slow folding conditions would also constitute evidence for a link between folding and glycosylation.

If we look specifically at the level of glycosylation at the 3T site, we see that it is not higher when DTT treatment and propeptide deletion are combined. In other words, the effects are not additive. This is the case for all mutants where DTT and propeptide deletion have an effect on the glycosylation level. It is noteworthy that the formation of local structure can be affected by DTT as well as by propeptide deletions. Furthermore, we did not expect that they would do so to such similar extents. We can, however, explain this finding by a model for the formation of the relevant local structure:




Under normal folding conditions, pathway A is dominant and along this pathway disulphide bonds are formed rapidly. Polypeptides that follow pathway A are not glycosylated at sequon 3T. If, on the other hand, disulphide bond formation is compromised, a sizeable fraction of the nascent polypeptides follow pathway B. Along this pathway, the 3T site is accessible to modification in a putative folding intermediate, I′. The model implies that, when folding is compromised by disruption of the propeptide, disulphide bonds are either formed less efficiently or with the wrong partners, resulting in the B pathway being favoured to an extent similar to that observed when disulphide bond formation is inhibited. In the absence of glycosylation of the sequon, both pathways A and B will eventually lead to the correctly folded structure.

Obviously, not all new sequons can be expected to conform to 'folding-dependent glycosylation', since this depends on their accessibility in folding intermediates and in the final folded structure. It is, for instance, fortuitous that the CPY-2 site just three amino acid residues N-proximal of the CPY-3 site is fully glycosylated irrespective of the conditions applied. This suggests that the location of the CPY-3 site in the helical structure of the 'disulphide zipper' does not *per se* prevent glycosylation. Modification of other sites has very negative affects on the folding (sequon 1S and 7S). Considering the buried nature of the introduced sequons, it is not surprising that in some of the mutants the overglycosylated precursors (3T, 4S and 4T) are eventually degraded (data not shown). Since it is not possible to exclude that the mutations introduced affect the rate of formation of local structure on their own, it is also obvious that introduction of glycosylation sites cannot be used as an absolute means for comparing the rate of folding of individual parts of the protein.

Our experiments do not address the question as to whether glycosylation could take place after complete translocation of the polypeptide. If not, it might, in principle, be possible to identify sites which are accessible in the folded structure but are inaccessible to the glycosyl transferase during folding. There are not many examples of glycoproteins which lack glycosylation at a specific sequon and for which the three-dimensional structure is also known. However, in the case of ovalbumin, the sequence Asn311–Leu–Ser is readily accessible on the surface of the protein (Stein *et al.*, 1991) and no proline residues are found in or around the sequon, which could influence glycosyl transfer. Nevertheless, this sequon is not glycosylated (Nisbet *et al.*, 1981), and it is tempting to speculate that this is indeed an example of transient inaccessibility. This would imply that glycosylation cannot take place after folding has been completed.

*In vitro* analysis of short peptides has shown that the $k_{cat}/K_m$ is 40-fold lower for substrates containing threonine in the third position than for those containing serine (Bause and Legler, 1981). Our mutants clearly show that this preference also holds true for several positions in the molecule in an *in vivo* context.

The relationship between folding, disulphide bond formation and glycosylation has been investigated most extensively using influenza virus haemagglutinin. Using very short pulse-labelling times, it is possible to label the nascent polypeptide chain and observe the modifications taking place in the nascent chain under different conditions (Chen *et al.*, 1995). In those studies, it was found that glycosylation took place rapidly after translocation of the sequons, when ~10 additional amino acid residues had passed through the membrane. This corresponds fairly well to the 12–14 residues found in an *in vitro* translocation–modification system (Nilsson and von Heine, 1993). This would leave time and space for the formation of secondary structure and interaction with more N-proximal parts of the polypeptide chain. *In vitro* studies have shown that the acceptor peptide has to adopt a specific conformation, the AsX turn, to become glycosylated (Imperiali *et al.*, 1992). Formation of alternative secondary structure forced by a change in the folding pathway could disrupt such a conformation. Recent studies on the tissue-type plasminogen activator have shown that conditions preventing disulphide bond formation lead to complete glycosylation of the otherwise partially utilized Asn184–Gly–Ser sequon (Allen *et al.*, 1995). This agrees well with our findings and supports the idea that there is a similar competition between folding and *N*-glycosylation in the ER also in higher eukaryotes.

Using mutants lacking several or all of the four normal

**Table III.** Oligonucleotides used in this study

| Oligo name | Sequence (5′→3′)[a] |
|---|---|
| oBH15 | G988GC TTG A**A**C GAG TCG TGC |
| oBH16 | G1052T AAT AAC **T**CC CAA TTG G |
| oBH17 | A1057AC GCC **AA**C TTG **T**CT CCT TAC |
| oBH18 | C1150AA GAT A**A**C GAC **AG**C TAC TTA AAC |
| oBH19 | C1239AA CAG AA**G** **C**TT CCT GTT TG |
| oBH20 | C1282AC ACC **AA**C GTA AC**C** GAT CTT |
| oBH21 | G1291TA ACA GAT **AA**T TTG A**G**T CAA GAC |
| oBH33 | C1150AA GAT A**A**C GAC **AC**C TAC TTA AAC |
| oBH34 | C1230TT CGA TAT **T**AA CAG AA**C** **C**TT CCT GTT TGC G |
| oBH35 | C1276CT TAC CAC ACC **AA**C GTA **TC**C GAT CTT TTG AAT C |
| oBH38 | A1054AT AAC GCC **AA**C TTG **A**CT CCT TAC CAA CG |

[a]Numbers indicate positions of the nucleotides in the *PRC1* open reading frame (Valls *et al.*, 1987). Nucleotides in bold indicate introduced mutations.

glycosylation sequons, it has been shown that, although the normal glycosylation of CPY is not essential for folding, it does aid the folding process in the ER (Winther *et al.*, 1991; te Hessen and Aebi, 1994). In view of the present work, such a linkage becomes even more obvious, since the similar time scale at which folding and glycosylation take place clearly provides an opportunity for the latter to drive later and more complex parts of the folding reaction. Furthermore, the approach described here offers a new method for assaying protein folding in the ER *in vivo*.

## Materials and methods

### Strains

The *Saccharomyces cerevisiae* strains used were JHRY20-2C (*MAT***a** *his3-Δ200 ura3-52 leu2-3,112 prc1Δ::HIS3*; Valls *et al.*, 1987), TSY8-10C (*MAT***a** *ura3 leu2 prc1::LEU2 sec18-1*, kindly provided by T.Stevens, University of Oregon) and RSY281 (*MAT***a** *his4-619 ura3-52 sec23-1*; Kaiser and Schekman, 1990, kindly provided by M.Knop, University of Stuttgart). *PRC1* was disrupted in RSY281 by gene disruption (Scherer and Davis, 1979) using plasmid pWI-17 (described below). The *Escherichia coli* strain DH5α (Sambrook *et al.*, 1989) was used for plasmid propagation; BMH71-18*mutS* (Zell and Fritz, 1987) and JM109 (Yanisch-Perron *et al.*, 1985) were used for site-directed mutagenesis.

### Media and materials

Yeast cells were grown in standard YPD and SC medium (Sherman, 1991). *E.coli* was grown in LB, SOC and 2× YT medium (Sambrook *et al.*, 1989). Restriction enzymes, T4 DNA polymerase, T4 polynucleotide kinase, T4 DNA ligase and Klenow polymerase were from Promega, Madison, WI. *Pfu* DNA polymerase was from Stratagene, La Jolla, CA. *Taq* DNA polymerase was from Perkin Elmer Corp. [$^{35}$S]Methionine was from DuPont NEN. Zymolase 100-T was from Seikagaku Kogyo, Tokyo, Japan. Fixed *Staphylococcus aureus* cells were IgGsorb from The Enzyme Center, Malden, MA. Tunicamycin was from Boehringer Mannheim. Oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer, desalted on NAP-5 columns (Pharmacia Biotech Inc.) equilibrated with 10 mM Tris–HCl, 1 mM EDTA, pH 8.0, and used as primers for PCR and sequencing reactions. Sequencing was performed using a *Taq* Dye Deoxy™ terminator cycle sequencing kit on an API 373A DNA Sequencer, both from Applied Biosystems, Foster City, CA.

### Plasmid construction and mutagenesis

Subcloning and transformation of *E.coli* and yeast were carried out using standard procedures (Ito *et al.*, 1983; Sambrook *et al.*, 1989). Plasmids used in this study, the resulting *PRC1* alleles, the encoded enzymes and the introduced amino acid changes are listed in Table I. Oligonucleotides used for mutagenesis are listed in Table III. Plasmid pWI-17 was constructed as follows: plasmid pWI-3 (Winther *et al.*, 1991) was digested with *Eco*RI and religated to remove the 2 μm segment. Subsequently, most of the mature region of *PRC1* was removed by digestion with *Bam*HI followed by religation. Plasmid pBH575 was constructed by digesting plasmid pJW1433 (Ramos *et al.*, 1994) with *Bam*HI and religation. Plasmid pBH584 was constructed as follows: pCRR-312 (Ramos *et al.*, 1994) was digested with *Sma*I followed by religation to give pBH566. This construct was then digested with *Bam*HI and religated. Site-directed mutagenesis was performed as described previously (Lewis and Thompsen, 1990) with the modification described by Olesen and Kielland-Brandt (1993). The mutations listed in Table I were generated in plasmid pYSP1 (Olesen and Kielland-Brandt, 1993), followed by subcloning as *Bam*HI–*Bam*HI fragments into either pBH575 or pBH584.

### Pulse-labelling and immunoprecipitation

Pulse-labelling and immunoprecipitation were performed essentially as described by Winther *et al.* (1991). Yeast cells were grown in SC-ura and starved for sulfur in SC-ura without $(NH_4)_2SO_4$. [$^{35}$S]Methionine was used for labelling instead of [$^{35}$S]$H_2SO_4$. When indicated, tunicamycin and DTT were added to give final concentrations of 10 μg/ml and 20 mM, respectively. In the mutants where rapid degradation took place (4S, 4T and 6S), the level of overglycosylation was monitored in *sec23* or *sec18* strains. Others were monitored at time points where all normally glycosylated p2-form had been processed in the vacuole.

## Acknowledgements

We are grateful to Birthe Kragelund for assistance with preparation of Figures 1 and 6 and for critical reading of the manuscript. Bart van den Hazel and David Simpson are thanked for critical reading of the manuscript and for helpful discussions. Ann-Sofi Steinholtz is acknowledged for preparation of photographic material. We thank Kjeld Olesen and Klaus Breddam for making results available prior to publication. This work was supported by the Danish Medical Research Council (J. nr. 12-2037-1).

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*Received on January 4, 1996; revised on March 15, 1996*