Peptide Binding by Protein Disulfide Isomerase, a Resident Protein of the Endoplasmic Reticulum Lumen*

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Previously we had demonstrated by photoaffinity labeling that a 57-kDa protein of the endoplasmic reticulum can bind and become covalently linked to glycosylated photoaffinity peptides containing the sequence -Asn-Xaa-Ser/Thr-. Subsequently, it was found that this protein, called glycosylation site-binding protein, was a multifunctional protein, i.e. it was identical to protein disulfide isomerase (PDI), the 1.7-subunit of prollyl hydroxylase and thyroid hormone-binding protein. In this study, the peptide specificity for binding to this 57-kDa protein, hereafter called PDI, has been investigated in more detail using photoaffinity probes. The results reveal that although N-glycosylation by oligosaccharyl transferase in the endoplasmic reticulum has an absolute requirement for an hydroxyamino acid in the third amino acid residue of the glycosylation site sequence, no such specificity is observed in the binding of such peptides to PDI. In addition to the lack of specificity for an hydroxyamino acid in the third residue position, no specificity was observed for the asparagine residue in the first position. Thus, binding is not restricted to peptides containing N-glycosylation sites. We have investigated the discrepancy between this apparent lack of specificity and earlier results indicating that binding of peptides to PDI was specific for N-glycosylation site sequences. We now demonstrate that PDI in the lumen of microsomes is more efficiently labeled by peptides containing photoaffinity -Asn-Xaa-Ser/Thr- sequences than by nonacceptor site sequences because the former become glycosylated. This increased labeling does not occur because the glycosylated form of the probes are preferentially recognized by PDI. Rather, it appears that increased polarity of the affinity probe after attachment of the oligosaccharide chain prevents its exit from the sealed microsomes, in effect concentrating it within the lumen of the microsome. These results, coupled with other studies on the multifunctional nature of PDI, suggest that the observed peptide binding may be a manifestation of the ability of PDI to recognize the backbone of polypeptides in the lumen of the endoplasmic reticulum.

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A considerable amount of information has been collected concerning the sequence requirements for the N-glycosylation of glycoproteins. An examination of the amino acid sequences adjacent to the asparagine residues bearing the oligosaccharide in N-linked glycoproteins led to the proposal that a serine or threonine must be 2 residues from the asparagine in order for glycosylation to occur (Marshall, 1974); i.e. the structure must be -Asn-Xaa-Ser/Thr-. Bause and Legler (1981) have suggested that these serine or threonine residues are involved in the catalytic activity and in support of this idea have shown that N-glycosylation site peptides containing the epoxy derivative of threonine are irreversible inhibitors of oligosaccharyl transferase activity. A further survey of the N-glycosylation site sequences has indicated that any of the 20 amino acids can occur in the -Xaa-position sequence, although some may be found less frequently than others (Kaplan et al., 1987). Because the carboxyamido side chain of the asparagine is involved in the formation of the N-glycosidic linkage to the oligosaccharide chain, it seemed obvious that modifications to that asparaginyl residue in acceptor peptides would preclude glycosylation. Indeed, experiments with glycosylation acceptor site tripeptides containing N-methyl asparagine or glutamine in place of asparagine revealed that these peptides were neither substrates for nor competitive inhibitors of oligosaccharyl transferase (Welply et al., 1983). Studies on the overall hydrophobicity of glycosylation site peptides revealed that modifications that increased hydrophobicity decreased the apparent Ks of the peptide (Welply et al., 1983). However, the results of these studies are of limited value with respect to substrate specificity; given the topology of the oligosaccharyl transferase reaction in the ER, the lower apparent Ks for more hydrophobic peptides may relate more to their increased delivery across the membrane than their specificity per se as a substrate.

As part of an effort to better understand the N-glycosylation reaction we undertook to identify the enzyme involved in this process, oligosaccharyl transferase, by developing a labeled photoaffinity probe containing an acceptor site tripeptide. Photolysis of this probe in the presence of microsomes resulted in labeling of a 57-kDa microsomal protein. However, this protein was found to be a resident protein of the lumen of the ER, rather than a membrane protein (Geetha-Habib et al., 1988). Because of its apparent specificity in binding peptides containing N-glycosylation sites and its luminal location, it was postulated that this protein, called glycosylation
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site-binding protein (GSBP), might participate in the N-glycosylation process (Geetha-Habib et al., 1988). However, studies by Bulleid and Freedman (1990) and our laboratory (Noiva et al., 1991) exclude obligatory participation since they indicate that depletion of proteins from the lumen of the ER does not impair N-glycosylation.

Initially GSBP was isolated from hen oviduct microsomes and purified by two-dimensional gel electrophoresis (Kaplan et al., 1988). Following preparation of antibody to the protein, a Agt11 expression library was screened and a cDNA clone expressing this protein was isolated. Sequence analysis (Geetha-Habib et al., 1988) of the cDNA encoding GSBP demonstrated high homology between hen oviduct GSBP and another luminal ER protein, protein disulfide isomerase (PDI). Protein disulfide isomerase has been proposed to function as the catalyst of disulfide bond rearrangement during post-translational folding based on its ability to accelerate the in vitro reactivation of denatured pancreatic ribonuclease (Goldberger et al., 1963) and its ability to catalyze the formation of intramolecular disulfide bridges during the in vitro biosynthesis of γ-gliadin (Bulleid and Freedman, 1988). On the basis of work from several laboratories it is now clear that GSBP is identical to PDI (Parkkonen et al., 1988; Kimura et al., 1990; LaMantia et al., 1991) and that it is a multifunctional protein that also is identical to the β-subunit of prolyl hydroxylase (Koivu et al., 1987), thyroid hormone-binding protein (Cheng et al., 1987), and dehydroascorbate reductase (Wells et al., 1990). Hereafter, for simplicity we term this protein PDI.

Given the above findings, we have undertaken a further investigation of the specificity of various peptides in binding to PDI and their ability to serve as substrates for N-glycosylation by oligosaccharyl transferase. The results of this study reveal that, in contrast to the glycosylation process, the binding of peptides to luminal PDI exhibits little specificity for the amino acid side chains. Further, they indicate that the apparent specificity for the binding of glycosylatable peptide probes by PDI is due to the fact that the glycosylated form of these probes, in contrast to non-glycosylatable probes, can be enriched in the lumen of the ER. The possible functional significance of peptide binding by PDI in the lumen is discussed.

MATERIALS AND METHODS AND RESULTS

DISCUSSION

We had previously demonstrated by affinity labeling that PDI, a multifunctional protein of the endoplasmic reticulum, binds peptides containing -Asn-Xaa-Ser/Thr- glycosylation sites. In the current study, we have extended these investigations to include a variety of other peptides similar to the N-glycosylation site photoaffinity probe, but with single amino acid substitutions for the asparagine or threonine. First, we have confirmed that the sequence -Asn-Xaa-Ser/Thr- is required for these photoprobe to be substrates for oligosaccharyl transferase. Second, we found no such sequence specificity for the binding of these peptides to PDI. Neither hydrophobic (-Thr- to -Phe-) nor hydrophilic (-Thr- to -Asp-) amino acid substitutions abolished peptide affinity probes recognition by PDI. Although the range of amino acid substitutions for the asparagine was limited (-Asn- to -Ala- or -Asp-), the results indicated that the substitutions resulted in probes that still labeled PDI but were not substrates for oligosaccharyl transferase. Based on these observations, we concluded that the ability of PDI to bind peptides is not limited to those containing N-glycosylation acceptor site sequences. This conclusion is consistent with recent findings that PDI, formerly called glycosylation site-binding protein, is not an obligatory component of the N-glycosylation process catalyzed by oligosaccharyl transferase (Bulleid and Freedman, 1990; Noiva et al., 1991).

Because we were unable to identify any specific amino acid requirements for the binding of peptides to PDI, we considered the possibility that affinity of the probe for PDI was related to either the 4-hydroxy-5-iodo-phenylpropionyl (Bolton-Hunter) group attached at the NH₂ terminus or the benzoylazido group attached to the ε-amino group of the lysine residue in the -Xaa- position. The former possibility was excluded by the initial observation that the affinity probe containing an [¹H]acetyl- group at the NH₂ terminus rather than the Bolton-Hunter moiety also specifically labels PDI (Welply et al., 1985). To investigate the latter possibility, we used compounds related to the p-azidobenzoyl group as competitors for binding of labeled NKT probe to PDI. In no case did these compounds block affinity labeling of PDI. We conclude therefore that neither these aromatic substituents, nor the specific amino acid side chains, serve as primary determinants for binding to PDI. Given the lack of amino acid side chain specificity it was not surprising to find that PDI was capable of binding the affinity probe before or after it was N-glycosylated. Consistent with this observation, the free core oligosaccharide or partial structures from the free core oligosaccharide did not inhibit the ability of PDI to bind to the N-glycosylated peptide. With respect to these findings, it is of interest to note that Flynn et al. (1989) have studied peptide binding by another luminal ER protein, BiP. Although differences in their individual peptides were detected, it was not possible to identify any specific sequence requirements for their binding by BiP. It is clear, however, that the binding process with BiP differs in that ATP is involved; we find no effect of ATP on peptide binding to PDI (data not shown).

Another major objective of these studies was to reconcile the apparent discrepancy between the lack of sequence specificity for peptide binding by free PDI with our earlier reports indicating peptide binding to PDI in the lumen of the microsomes is specific for peptides with N-glycosylation sites. Our findings indicate that affinity probes containing the N-glycosylation acceptor site sequence preferentially label PDI because of their ability to become glycosylated and thereby become entrapped in the lumen. Consistent with this idea it was found that glycosylation of the affinity probe was found to enhance affinity labeling of PDI only when intact microsomes were used. Indeed, earlier it was demonstrated that upon washing of the membranes the majority of the glycosylated tripeptide remained associated with microsomal vesicles, but 99% of the unglycosylated tripeptide did not (Welply et al., 1983). This result suggested that the increased hydrophilicity of the glycosylated peptide precludes movement across the microsomal membrane, thereby effectively concentrating it in the lumen relative to the unglycosylated probe. We have now demonstrated that, in fact, the glycosylated photoaffinity probe is unable to cross the microsomal membrane. We conclude therefore that the much higher level of labeling of PDI by glycosylatable peptides occurs because glycosylation serves as a “sink” to entrap the glycopeptide in the lumen. As a consequence the glycosylated peptide labels PDI better than non-glycosylatable probes because it is present at much higher concentrations, not because it has special structural features.

2 Portions of this paper (including “Materials and Methods,” “Results,” Figs. 1–8, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
preferentially recognized by PDI.

The results of these studies indicate that PDI binds peptides but lacks any clearly definable sequence specificity. As noted earlier, this 57-kDa luminal protein has been demonstrated to be identical to 1) the β-subunit of prolyl hydroxylase (Koivu et al., 1987), an enzyme that catalyzes the post-translational modification of collagen, 2) thyroid hormone-binding protein (Kimura et al., 1990), and 3) dehydroascorbate reductase (Wells et al., 1990). The peptide binding activity we have detected may be directly involved in one of these other functions of PDI. However, several lines of preliminary evidence argue against this idea. First, a peptide substrate for prolyl hydroxylase, (Pro-Pro-Gly), did not compete with the peptide probes used in these studies for affinity labeling, suggesting the collagen and PDI peptide-binding sites are different. Second, the active site that recognizes the collagen polypeptide chain is on the α-subunit of prolyl-hydroxylase, not the β-subunit (de Waal et al., 1985). Third, the peptide binding activity of the 57-kDa protein does not appear related to its function as PDI because alkylation of the cysteine pairs in -CGHC- sequences in the proposed active sites of PDI inactivated isomerase activity but had no effect on peptide binding. Fourth, neither thioredoxin nor the β-subunit of chorionic gonadotropin, two other proteins containing the proposed -CGHC- active sites of PDI are labeled by the peptide probe. Finally, it is important to note that labeling of PDI by peptide probe is not merely a consequence of the fact that it is present in high concentration (0.4% of total cellular protein) but other luminal proteins such as BIP are present in similar concentrations yet are not labeled. Thus, this luminal protein uniquely recognizes peptides, although we have been unable to discern primary sequence requirements. Perhaps this binding is a manifestation of a common first step, perhaps a type of editing process, that must occur prior to the various other processes in which this multifunctional protein participates.

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REFERENCES


3 R. Noiva and W. J. Lennarz, unpublished observations.
4 R. Noiva and W. J. Lennarz, manuscript in preparation.


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**Peptide Binding by Protein Disulfide Isomerase**

**TABLE II**

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>CON. RANGE (nmol)</th>
<th>PHOTODENSING IN % OF CONTROL</th>
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<tr>
<td>GlcN Ac</td>
<td>3.7-13.0</td>
<td>100</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>0.05-1.0</td>
<td>100</td>
</tr>
<tr>
<td>Man &amp; GlcNAc &amp; Asn</td>
<td>0.05-1.0</td>
<td>100</td>
</tr>
<tr>
<td>Man &amp; GlcNAc &amp; Asn</td>
<td>0.025-1.0</td>
<td>100</td>
</tr>
</tbody>
</table>

*The control contained no added oligosaccharide.*

**Fig. 1** Structure of the photolabeling probe, N\(^{25}\)(3Me)\(2-(\beta\)-D-glucopyranosylpropionyl)-Lys-Ago-2-picolinamide (Ag-NH\(_2\)).

**Fig. 2** Peptide photolabeling probes containing N-substituent for the threonine or asparagine residues of the glycosylation site were recognized and bound by PDI. Long-time exposure to the 100% concentration of glucose added with the indicated amounts of photolabels following 2 min postaddition. A) PDI was affinity labeled with \(\beta\)-Ago-Lys(Lys\(_{2}\))-Thr-NH\(_2\) (NKT probe), \(\beta\)-Ago-Lys(Lys\(_{2}\))-Asp-NH\(_2\) (NKT probe), \(\beta\)-Ago-Lys(Lys\(_{2}\))-Thr-NH\(_2\) (NKT probe), or \(\beta\)-Ago-Lys(Lys\(_{2}\))-Asp-NH\(_2\) (NKT probe). B) PDI was affinity labeled with NKT probe, NKA probe, or \(\beta\)-Ago-Lys(Lys\(_{2}\))-Thr-NH\(_2\) (NKT probe). The lower panel shows the SDS-PAGE of the complexes formed with affinity-labeled micromolecules.

**Fig. 3** Peptide inhibition of affinity labeling of PDI with NKT probe does not depend on the presence of a N-glycosylation site sequence. (A) PDI was affinity labeled with \(\beta\)-Ago-Lys(Lys\(_{2}\))-Thr-NH\(_2\) (NKT probe), in the presence of a non-reducible peptide, or \(\beta\)-Ago-Lys(Lys\(_{2}\))-Thr-NH\(_2\) (NKT probe), in the presence of a reducible peptide. (B) PDI was affinity labeled with \(\beta\)-Ago-Lys(Lys\(_{2}\))-Thr-NH\(_2\) (NKT probe), in the presence of a non-reducible peptide, or \(\beta\)-Ago-Lys(Lys\(_{2}\))-Thr-NH\(_2\) (NKT probe), in the presence of a reducible peptide. (C) PDI was affinity labeled with \(\beta\)-Ago-Lys(Lys\(_{2}\))-Thr-NH\(_2\) (NKT probe), in the presence of a non-reducible peptide, or \(\beta\)-Ago-Lys(Lys\(_{2}\))-Thr-NH\(_2\) (NKT probe), in the presence of a reducible peptide. Affinity labeling was measured by autoradiography of the labeled SDS-PAGE of separation on SDS-PAGE.

**Fig. 4** Mn\(^{2+}\) was required for glycoprotein labeling. PDI (100 μg) was photolabeled (open bars) in 0.5 M Tris-Cl buffer, pH 7.3, with the indicated steady-state concentrations of mercaptoethanol. Glycoprotein of acceptor peptide (open bars) was measured using reduced micromolecules (100 μg) in glycoprotein buffer, pH 7.3, using the indicated concentrations of mercaptoethanol.

**Fig. 5** Affinity probes lacking the N-glycosylation site sequence did not label PDI in the absence of micromolecules in the same manner as a probe containing the glycosylation site. PDI-bound micromolecules were affinity labeled with probe incubated with varying quantities of \(\beta\)-Ago-Lys(Lys\(_{2}\))-Thr-NH\(_2\) (NKT probe), \(\beta\)-Ago-Lys(Lys\(_{2}\))-Asp-NH\(_2\) (NKA probe), or \(\beta\)-Ago-Lys(Lys\(_{2}\))-Thr-NH\(_2\) (NKT probe) in the presence of 10 μM Mn\(^{2+}\). The autoradiograph of the affinity-labeled micromolecules after separation on SDS-PAGE and the relative intensities of the bands are shown.

**Fig. 6** Affinity labeling of microsomal PDI with glycosylation site acceptor and non-acceptor probes. Microsomal micromolecules were incubated with \(\beta\)-Ago-Lys(Lys\(_{2}\))-Thr-NH\(_2\) (NKT probe) or \(\beta\)-Ago-Lys(Lys\(_{2}\))-Asp-NH\(_2\) (NKA probe) in the presence of 10 μM Mn\(^{2+}\) of EDTA. The bands corresponded to 75000 Da after autoradiography, as well as their relative intensities, are shown.

**Fig. 7** Affinity labeling with a photolabeling probe. Probe, glycosylated probe, and enzymatically digested and photolabeled probe (86 kDa) were used to label the labeled proteins (150 μg) of complex micromolecules. Lane 1, Labeled with n-glycosylated probe, lane 2, PDI labeled with control glycosylated probe, lane 3, PDI labeled with reduced glycosylated probe. The bands corresponding to 75000 Da (75000 kDa) as well as their relative intensities at 57 kDa.

**Fig. 8** Glycosylated photolabeling probe does not cross the microsomal membrane. Freshly prepared micromolecules were incubated with a n-glycosylated (left lane) or glycosylated (right lane) N-glycosylated acceptor site photolabeling probe for 20 min, and then photolabeled. Labeled micromolecules were then separated free probe by ultracentrifugation over a sucrose cushion. The amount of affinity labeling was then determined by autoradiography of the micromolecules after separation on SDS-PAGE. Both the control (A) and autoradiography (B) are presented.