Letter to the Glyco-Forum

Deglycosylation of hen ovotransferrin under mild conditions: effect on the immunoreactivity and biological activity

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Transferrins belong to a group of homologous iron-binding glycoproteins present in all vertebrates (for review see: Loehr, 1989; Crichton, 1991). Main forms of this protein include serum transferrin from different species; ovotransferrin from avian egg white and lactotransferrin from mammalian milk (Aisen and Listowsky, 1980). These glycoproteins possess a single polypeptide chain with a molecular weight of about 80 kDa folded up to give two similar lobes, N- and C-terminal, each with a single binding site for Fe(III) (Aisen and Listowsky, 1980). Remarkable differences among transferrins can be seen taking into account the carbohydrate moiety, the so-called glycan, attached to the polypeptide backbone. In fact, whereas both human serum transferrin and lactotransferrin present two N-glycans, exclusively located in the C-lobe in human serum transferrin and symmetrically distributed in lactotransferrin, hen ovotransferrin possesses a single N-glycan at the C-lobe (Metz-Boutigue et al., 1984). Moreover, the glycans of most serum transferrin and lactotransferrin are mainly biantennary and terminating in stialic acid residues (Spik et al., 1975, 1982), but interestingly, the glycan of hen ovotransferrin shows a tetra-antennary structure terminating in N-acetylglucosamine (Dorland et al., 1979). As found for transferrins (De Jong et al., 1989; Léger et al., 1989) and many other glycoproteins (Kobata, 1992), glycans could be involved in recognition mechanisms, and in the case of transferrins also in iron delivering (Bomford and Munro, 1985). Being in most instances interested in the purification and characterization of glycans, deglycosylation is usually carried out in harsh conditions for the protein moiety; but in many other cases, where it is necessary to understand the role of glycans in protein structure/function relationship, deglycosylation should be performed in conditions “mild” enough to keep glycoproteins in their native or active state. Thus, in the present paper, a method we already described (D’Andrea et al., 1993), was adopted to obtain exhaustively deglycosylated hen ovotransferrin in “mild” denaturing conditions. Results were then compared with two other standard deglycosylation approaches both based on harsh conditions, i.e., the chemical method reported by Edge et al. (1981), and the “hard” standard enzymatic approach.

As shown in Figure 1A, when samples were stained with Coomassie brilliant blue, a main band could be seen, with an apparent molecular weight compatible with that of the full-length hen ovotransferrin (OTf) molecule, purified according to Williams (1968). This apparent molecular mass shifted from ∼80 kDa (Figure 1A, lanes 2, 5–9) to ∼78 kDa (Figure 1A, lanes 3, 4, 10). Thus, efficient deglycosylation was achieved when OTf was treated either with trifluoroethanesulfonic acid (TFMS), or in “hard” enzymatic conditions, or preincubated with 0.1% (w/v) SDS + 0.5% (v/v) NP-40 (D3) and subsequently treated with peptide N-glycosidase F (PNGase F). This finding was confirmed by lectin blotting: when a duplicate gel was probed with concanavalin A (Con A), a lectin which recognizes both branched mannose and β-N-acetylglucosamine residues, only a faint staining appeared when the sample was subjected to “hard” enzymatic conditions (Figure 1B, lane 4) or was preincubated with detergent mixture D3 and then subjected to digestion with PNGase F (Figure 1B, lane 10). A still fainter band appeared when the sample was chemically treated (Figure 1B, lane 3). Positive staining for lanes 5–9 (Figure 1B) indicated that N-linked side chain was still bound to OTf. Parallel behavior was observed when samples were analyzed by immunoblot. In these experiments, all the OTf samples were recognized by polyclonal anti-native OTf antibodies, obtained following the procedure reported by Campbell et al. (1974), but the sample treated with TFMS (Figure 1C, lane 3), the sample subjected to “hard” enzymatic conditions (Figure 1C, lane 4), and the sample preincubated with 0.1% (w/v) SDS + 0.5% (v/v) NP-40 (D3) and then subjected to digestion with PNGase F (Figure 1C, lane 10) all gave a faint positive band. As determined by the Con A binding assay (Rhodes and Ching, 1993), the much more efficiently deglycosylated OTf forms, that is, the sample TFMS-treated, the one treated in “hard” enzymatic conditions, and the sample treated in “mild” enzymatic conditions (detergent mixture D3) underwent a 98%, 92%, and 87% (w/w) of glycan removal, respectively (Table I). Moreover, binding studies showed that the maximum value of bound 125I-OTF molecules per cell (Bmax) was essentially the same being about 3.15 × 10⁵ for native OTF and 1.13 × 10⁴ for OTF deglycosylated in “mild” conditions, respectively (Table II). Iron delivering, measured as ⁵⁹Fe atoms delivered per cell per min did not appreciably change for both native and “mildly” deglycosylated OTF (1.28 × 10³ and 1.13 × 10⁴, respectively; Table II). The other deglycosylated OTF forms were also tested but no one was able neither to bind nor to deliver iron to chick-embryo red blood cells (CERBCs) (data not shown).

Conclusions
It is now accepted that glycan(s), that is the carbohydrate side chain(s), in glycoconjugates can play different fundamental biological roles (Rademaker et al., 1988); thus, complete carbohydrate removal is often desirable, but difficult, because the secondary and tertiary structures of the polypeptide chain may...
obstruct or prevent the enzymatic action of glycosidases. In the case of OTI, exhaustive deglycosylation under conditions mild

enough was particularly challenging. In particular, to better investigate in the future the role of the glycan in OTI, preincubation with three different detergent mixtures (1 h, 37°C) was performed before enzymatic deglycosylation (24 h, 37°C). However, in a first attempt we tried to deglycosylate OTI with 4–10 U PNGase F at 37°C for 12–48 h, but samples were found strongly positive when stained after Con A blot, indicating the continued presence of glycans in our glycoprotein (data not shown). Then, different detergents were chosen as single or as mixture as being likely to keep PNGase F highly active (Haselbeck and Hösel, 1988). Thus, an efficient removal of N-glycans was obtained only when D3 mixture was added to the incubation medium prior to the PNGase F (Figure 1A–C, lane 10). Nevertheless, in other similar deglycosylation studies, a different detergent mixture (Long et al., 1991) or stoichiometric amounts of PNGase F (Langer et al., 1987) have been found to be successful. Anyway, for our purpose we tried also other experimental “mild” conditions (mainly different incubation times, and different amounts of PNGase F), but in the present study conditions which gave the best results in the shortest incubation time and with the minimum amount of PNGase F, have been reported.

### Table I. Release of N-linked glycan from OTI as measured by the Con A binding assay

<table>
<thead>
<tr>
<th>Glycoconjugate</th>
<th>Residual glycosylation (%)</th>
<th>Concentration of coated substrate (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-OTI</td>
<td>2</td>
<td>530</td>
</tr>
<tr>
<td>H-OTI</td>
<td>8</td>
<td>510</td>
</tr>
<tr>
<td>M-OTI</td>
<td>13</td>
<td>500</td>
</tr>
</tbody>
</table>

### Table II. Relevant parameters of binding (B_{max}) and iron delivering to CERBCs

<table>
<thead>
<tr>
<th>Glycoconjugate</th>
<th>B_{max} (×10^{-5})</th>
<th>Iron delivering (×10^{-5})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native OTI</td>
<td>3.15</td>
<td>1.28</td>
</tr>
<tr>
<td>M-OTI</td>
<td>2.95</td>
<td>1.13</td>
</tr>
</tbody>
</table>

For binding studies, CERBCs collected from 14-day-old chick-embryos were prepared and treated as reported previously (Mason et al., 1987; D’Andrea et al., 1995) and put in the presence of ^{125}I-OTI at various concentrations (1–50 µM). ^{125}I-Labeling was performed according to Oratore et al. (1989) with specific activity ranging from 5.3 × 10^{6} to 6.1 × 10^{6} Ci/mmol of protein in different preparations. For iron delivering experiments, the experimental protocol adopted was essentially the same as previously reported (Williams et al., 1992; D’Andrea et al., 1995). ^{59}Fe-labeling protocol was as described previously (D’Andrea et al., 1995) with specific activity ranging from 50 to 100 Ci/mmol. M-OTI, OTI “mild” enzymatically treated (detergent mixture D3).

a Percent residual glycosylation (\([A_{np}/A_p] \times 100\)) was obtained by comparing the absorbance values (as a measure for affinity towards Con A) of samples at a coat concentration of 500 ng/ml before (\(A_p\)) and after deglycosylation (\(A_{np}\)).

b Values are nanograms of glycoconjugate/ml buffer yielding an absorbance of 0.5 at 495 nm wavelength under conditions of the test.
Interestingly, polyclonal anti-native OTf antibodies bound poorly to TFMS chemically-treated OTf, “hard” enzymatically-treated OTf, and D3-PNGase F-treated OTf (Figure 1C, lanes 3, 4, 10), hypothesizing that antibodies could mostly be directed against the N-linked oligosaccharide side chain of OTf, although a modification of protein conformation should be also envisaged after deglycosylation. Furthermore, since OTf glycan represents about 3% of the mass of the native protein (Spik et al., 1988), it appeared probable that these sugar anticogent determinants were strongly immunogenic.

In determining the degree of deglycosylation judged by Con A binding assay, even if both the “hard” enzymatic and the chemical approach were the most effective, they are to much detrimental for biological activity of OTf; moreover, following the method described by Edge et al. (1981) about 45% of protein was lost during the many steps the procedure requires (data not shown). In the case of enzymatic deglycosylations the removal of sugar was slight less efficient, but no loss of protein occurred, although in the case of “hard” conditions OTf looses its biological activity. Of course, best deglycosylating conditions should be tried and found for each specific molecule, but the “mild” approach we adopted in the present study and in the past (D’Andrea et al., 1993) could certainly be tested also for other glycoproteins or glycoconjugates.

Eventually, based upon our previous studies (Oratore et al., 1989, 1990; Williams et al., 1992), binding and uptake experiments here reported show, as well as appeared in a recent study (Hoefkens et al., 1997), that our OTf, deglycosylated in “mild” conditions, is able both to bind and to deliver iron to CERBCs indicating the retention of biological activity also when OTf is presented without its N-glycan. In particular, binding capacity, measured as $B_{\text{max}}$ for control cells was only 7% higher than OTf deglycosylated in “mild” conditions (3.15×10^5 versus 2.95×10^5, respectively; Table II); iron delivering, measured as 59Fe atoms delivered per cell per minute, was around 13% more for control OTf with respect to OTf deglycosylated in “mild” conditions (1.28×10^5 versus 1.13×10^5, respectively; Table II). Then, as already reported in the above cited paper (Hoefkens et al., 1997) where deglycosylated human transferrin is shown to be biologically active as well as the native form, it seems that the presence of carbohydrate side chain in OTf too do not represent a prerequisite for binding to its specific receptor and then deliver its iron.

Lastly, as in our previous study (D’Andrea et al., 1993), we report a procedure for deglycosylating native OTf under “mild” denaturing experimental conditions making use of detergent preincubation before enzymatic deglycosylation, in addition, compare the effect of three different deglycosylating approaches on the extent of deglycosylation, test the immunoreactivity of the deglycosylated forms of OTf, determine the degree of deglycosylation and, eventually, we show that OTf deglycosylated according to our “mild” approach is still able to keep most of its biological activity.

Acknowledgments
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Abbreviations
CERBC, chick-embryo red blood cell; Con A, Concanavalin A; NP-40, Nonidet P-40; OTF, hen ovotransferrin; PAGE, polyacrylamide gel electrophoresis; PNGase F, peptide-N-deacetyl-β-glucosaminidase F (E.C. 3.5.1.52), commonly known as peptide N-glycosidase F; SDS, sodium dodecyl sulfate; TFMS, trifluoromethanesulfonic acid.

References


Announcement

Dr. Akira Kobata will be stepping down as the Glycobiology Executive Editor for Japan and will be succeeded by Dr. Takashi Muramatsu of the Nagoya University School of Medicine. As of November 1, 1999, all authors in that region should submit manuscripts to Dr. Muramatsu at the following address:

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Meeting Announcements

GLYCO XV
XVth International Symposium on Glycoconjugates

Hotel East 21, Tokyo, Japan
August 22–27, 1999

Keynote Lecturer: R.Colin Hughes (UK)
Closing Lecturer: Stuart Kornfeld (USA)
Plenary Lecturers: Henrik Clausen (Denmark), Minoru Fukuda (USA), Carlos Hirschberg (USA), Toshikazu Kawasaki (Japan), Robert Rosenberg (USA), Pierre Sinay (France), Andrew P.Spicer (USA), Kunihiko Suzuki (USA)

Chairman: Akira Kobata
Vice Chairman: Toshiaki Osawa, Yoshiyuki Nagaï, Tomoya Ogawa
Chairman of Program Committees: Tatsuro Irimura

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The following satellite symposia will be held:

(I) International Conference on Molecular Interactions of Proteoglycans
Shonan Village Center, Kanagawa, Japan
August 27–30, 1999

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(II) New Frontier of Glyco- and Lipid-Biology Toward 21st Century
Vaga Hall in Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan
August 28–30, 1999

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(III) Molecular Immunology of Complex Carbohydrates II
Chang-Gung Medical College, Taiwan
August 28–31, 1999

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1999 Xiangshan Science Conference on Glycoconjugates and Human Health
Fragrant Hill (Xiangshan) Hotel, Xiangshan Park, Beijing, China
September 1–4, 1999

Xiangshan Science Conferences have been organized and sponsored by the Ministry of Science and Technology of China and the Chinese Academy of Sciences since 1993. The goal of the conference is to bring together scientists to discuss recent advances and trends in science research frontiers. The 1999 conference will concentrate on recent advances in the fields of glycochemistry, glycobiochemistry, and glycotechnology with regard to innovative approaches and applications in medicinal chemistry, health care, and basic research. Special emphasis will be given to the potentials and problems of human glyco-therapeutic substances from recombinant and natural sources, especially from traditional Chinese medicines. Analytical and synthetic methods and tools will also be of central importance.

Invited speakers include: R.Cummings (USA), R.Dwek (UK), A.Elbein (USA), T.Feizi (UK), S.-I.Hakomori (USA), S.Hanessian