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Site-specific *N*-glycan characterization of human complement factor H

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Human complement factor H (CFH) is a plasma glycoprotein involved in the regulation of the alternative pathway of the complement system. A deficiency in CFH is a cause of severe pathologies like atypical haemolytic uraemic syndrome (aHUS). CFH is a 155-kDa glycoprotein containing nine potential N-glycosylation sites. In the current study, we present a quantitative glycosylation analysis of CFH using capillary electrophoresis and a complete site-specific *N*-glycan characterization using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and liquid chromatography–electrospray ionization tandem mass spectrometry (LC-ESIMS/MS). A 17.9-kDa mass decrease, observed after glycosidase treatment, indicated that N-glycosylation is the major post-translational modification of CFH. This mass difference is consistent with CFH glycosylation by diantennary disialylated glycans of 2204 Da on eight sites. CFH was not sensitive to endoglycosidase H (Endo H) deglycosylation, indicating the absence of hybrid and oligomannose structures. Quantitative analysis showed that CFH is mainly glycosylated by complex, diantennary disialylated, non-fucosylated glycans. Disialylated fucosylated and monosialylated non-fucosylated oligosaccharides were also identified. MS analysis allowed complete characterization of the protein backbone, verification of the glycosylation sites and site-specific *N*-glycan identification. The absence of glycosylation at Asn_{199} of the NGS*P* sequence of CFH is shown. Asn_{511}, Asn_{700}, Asn_{784}, Asn_{804}, Asn_{864}, Asn_{893}, Asn_{1011} and Asn_{1077} are glycosylated essentially by diantennary disialylated structures with a relative distribution varying between 45% for Asn_{804} and 75% for Asn_{864}. Diantennary monosialylated glycans and triantennary trisialylated fucosylated and non-fucosylated structures have also been identified. Interestingly, the sialylation level along with the amount of triantennary structures decreases from the N- to the C-terminal side of the protein.

Keywords: complement factor H/glycosylation/mass spectrometry

Introduction

Human complement factor H (CFH) is a single-chain plasma glycoprotein of ~155 kDa (Rodriguez de Cordoba et al. 2004). The secreted form consists of 1213 amino acids distributed into 20 repetitive units of approximately 60 amino acids, called short consensus repeats (SCRs) (Ripoche et al. 1988). Its primary sequence is depicted in Figure 1.

CFH is essential for regulating the activation of the alternative pathway of complement and for restricting the action of complement to activating surfaces. In the early stage of the complement cascade, CFH can compete with factor B for binding to C3b, thereby preventing the formation of the C3 convertase C3bBb. CFH can also bind to the formed convertase and accelerate its dissociation of this complex (decay accelerating activity). Another role for CFH in down-regulating the alternative pathway is its action as a cofactor for the serine protease complement factor I (CFI), which cleaves C3b into inactive fragments (Weiler et al. 1976; Whaley and Ruddy 1976; Pangburn et al. 1977).

The activity of CFH is potentiated by sialic acid or glycosaminoglycans (GAGs) at cell surfaces (Kazatchkine et al. 1979). CFH is able to discriminate host from pathogen cell surfaces. In the presence of host cells, CFH is able to rapidly inactivate the C3bBb complex. Quantitative or qualitative deficiencies in CFH therefore induce an accumulation of C3bBb at host cell surfaces leading to their opsonization. These deficiencies are a cause of severe pathologies like atypical haemolytic uraemic syndrome (aHUS) (Taylor 2001; Fremeaux-Bacchi et al. 2005).

The interaction between CFH and partners seems to be predominantly ionic in nature. For instance, it has been shown that binding reactions between CFH, CFI and C3b are very dependent on ionic strength and pH (Soames and Sim 1997). The capacity of the alternative pathway of complement to discriminate between self ('non-activators') and non-self ('activators') structures is mainly based on the interaction between CFH, C3b and the surface where C3b is bound. The non-activator self-structure has been demonstrated to be rich in sialic acids and/or GAGs such as heparan sulphate. These anionic structures are thought to increase the affinity of CFH binding to C3b deposited on particles or cell surfaces, thereby negatively regulating complement activation on the surfaces (Kazatchkine et al. 1979; Meri and Pangburn 1990; Pangburn et al. 2000). Nevertheless, since CFH does not bind to cell surfaces expressing sialic acid in the absence of C3b, it is likely that the increased affinity between CFH and C3b is a consequence of the simultaneous recognition of both sialic acids and bound C3b by the same CFH molecule (Rodriguez de Cordoba et al. 2004). The presence of several binding sites for both C3b and polyanions in CFH (Zipfel et al. 2002) reinforces this concept. The capacity of CFH to interact with surface-bound C3b is lost after trypsin treatment (Alsenz et al. 1984) or guanidination of lysine residues (Jouvin et al. 1984), but not after desialylation or

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Fig. 1. Primary amino acid sequence of human CFH (P08603). N-glycosylation consensus motifs NxS/T are represented in bold. Identified tryptic peptides are underlined whereas additional amino acid residues identified after Asp-N cleavage are italicized and shadowed. The sequence is shown arranged in the 20 SCR homologous units.

deglycosylation (Jouvin et al. 1984). These different results suggest that the CFH N-linked glycans may have structural rather than functional roles in the interactions with their natural biological partners, by regulating the areas of protein surface exposed for protein–protein interactions instead of participating directly in the ionic interactions through charged sialic acids.

The site-specific characterization of CFH's N-linked glycans would be very useful in determining the precise structure of interacting areas. This would facilitate partial deglycosylation approaches for subsequent functional analysis, to further obtain more insight into the role of N-linked glycans in the mechanism of CFH's action.

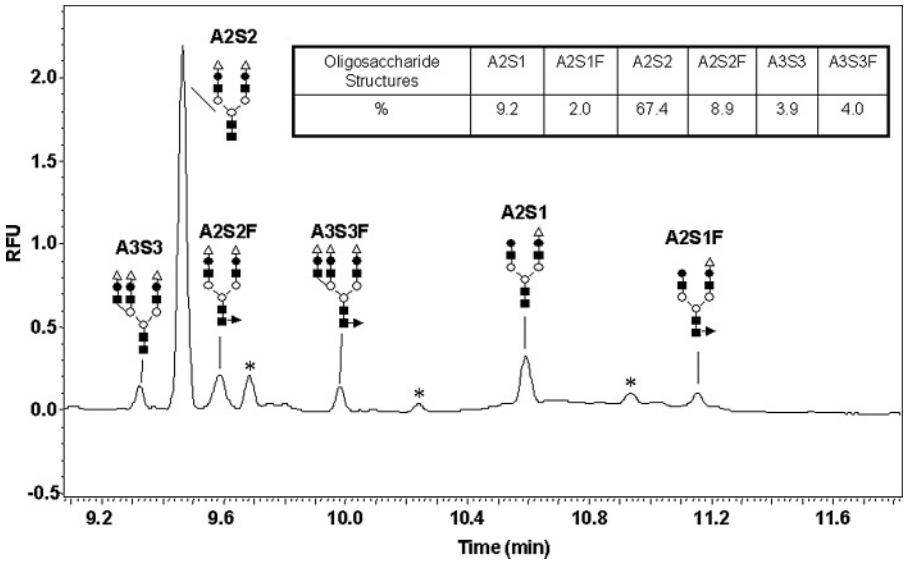
The N-glycosylation of CFH has not been well characterized. For instance, the reported carbohydrate content varies from 9.3% (Sim and DiScipio 1982) to 18.5% (Jouvin et al. 1984). CFH has nine potential N-glycosylation sites (Figure 1); these sites are located at Asn_{199}, Asn_{511}, Asn_{700}, Asn_{784}, Asn_{804}, Asn_{864}, Asn_{893}, Asn_{1011} and Asn_{1077}. Although no specific work has been dedicated to the study of CFH N-glycosylation, some data on site occupancy can be found in the literature. These data are somewhat controversial regarding the first N-glycosylation site, Asn_{199}. CFH-dedicated studies have first shown that Asn_{199} was unoccupied (Ripoche et al. 1988; Malhotra et al. 1999) whereas results from recent large-scale studies of plasma glycoproteins are either in favour of occupancy (Hagglund et al. 2004; Wang et al. 2006) or non-occupancy (Anderson and Hunter 2006; Zhang et al. 2007) of this specific site. The only available information on the composition of CFH N-linked glycans was obtained from the analysis of the total glycan pool of human CFH, and was reported to contain predominantly complex diantennary disialylated glycans (Ritchie et al. 2002). Regarding site-specific oligosaccharide heterogeneity, a single glycoproteomics study of human serum, through the use of a sialic-acid-specific lectin, has underlined the sialylation of glycans linked to Asn_{864} and Asn_{1011} (Qiu and Regnier 2005a). In fact, no dedicated site-specific study has been conducted on CFH.

The aim of the present study was to fully characterize the N-glycosylation of CFH. Highly efficient separation techniques, laser-induced fluorescence (LIF) detection, and various mass spectrometric (MS) measurements were combined to perform a comprehensive structural characterization of N-linked oligosaccharides of CFH. Oligosaccharide profiling and quantification were first performed by high-performance capillary electrophoresis with LIF detection (HPCE-LIF). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS), in combination with enzymatic deglycosylation, was then used to estimate the carbohydrate content of CFH and the number of occupied N-glycosylation sites. Finally, the glycosylation sites and their occupancy were precisely determined using a combination of enzymatic digestions, high-performance liquid chromatography (HPLC) separation, MALDI-TOF and TOF/TOF experiments in conjunction with electrospray (ESI-MS) and tandem mass spectrometry (MS/MS) analyses.

Results

CFH oligosaccharide quantification by HPCE-LIF

This approach provides valuable qualitative and quantitative data on the different CFH glycan structures. The HPCE-LIF electropherogram of *N*-glycans derived from CFH is depicted in Figure 2. The major oligosaccharides were complex diantennary and triantennary sialylated structures. Complex diantennary disialylated structures (A2S2) were found to be in large excess over the other sialylated glycans. Indeed, A2S2 structures accounted for 67% (mol/mol) of the total *N*-glycan pool of CFH, whereas diantennary monosialylated structures (A2S1)



Oligosaccharide Structures	A2S1	A2S1F	A2S2	A2S2F	A3S3	A3S3F
%	9.2	2.0	67.4	8.9	3.9	4.0

Fig. 2. HPCE-LIF electropherogram of APTS-labelled *N*-glycans derived from CFH. Relative proportions of the different structures are indicated in the accompanying table. Monosaccharide identification: (▲) fucose; (●) galactose; (■) GlcNAc; (○) mannose; (△) sialic acid. Peaks labelled with an asterisk correspond to sialylated structures.

accounted for only 9% (Figure 2). Beside these diantennary structures, triantennary trisialylated structures were observed and contributed to 8% (A3S3 and A3S3F, see Figure 2) of the total glycan pool. A small proportion of glycan structures was found to be fucosylated (F): A2S1F (2%), A2S2F (9%) and A3S3F (4%). Moreover, it should be noted that no oligomannose or hybrid structures were observed within the glycan pool.

CFH glycosylation examined by MALDI-TOFMS

An analysis of CFH before and after deglycosylation with specific enzymes provides valuable information regarding carbohydrate content, major glycan structures, as well as N-glycosylation site occupancy. For instance, Figure 3 shows the MALDI mass spectra obtained for native CFH, and CFH deglycosylated with either PNGase F or endoglycosidase H (Endo H). The molecular mass of native CFH was determined to be 154,882 Da (Figure 3A), and decreased to 136,931 Da after PNGase F treatment (Figure 3B). This value is in good agreement with the theoretical average molecular mass of 136,946 Da (considering 40 disulphide bridges) calculated from the amino acid sequence of CFH. This finding confirms that N-glycosylation is the major post-translational modification of CFH. The observed change in molecular mass from deglycosylation (∼17.9 kDa) indicates the presence of extensive N-linked glycosylation, accounting for ∼11.5% of the mass of native CFH. CFH was also treated with Endo H to estimate the contribution of high mannose and hybrid glycans. It was insensitive to Endo H (Figure 3C), which is consistent with the HPCE-LIF data indicating that CFH contains predominantly complex-type glycans. Moreover, considering that diantennary disialylated structures (∼2.2 kDa) are by far the most abundant ones, the molecular mass decrease of 17.9 kDa observed upon PNGase F treatment suggests that only eight among nine potential N-glycosylation sites are occupied.

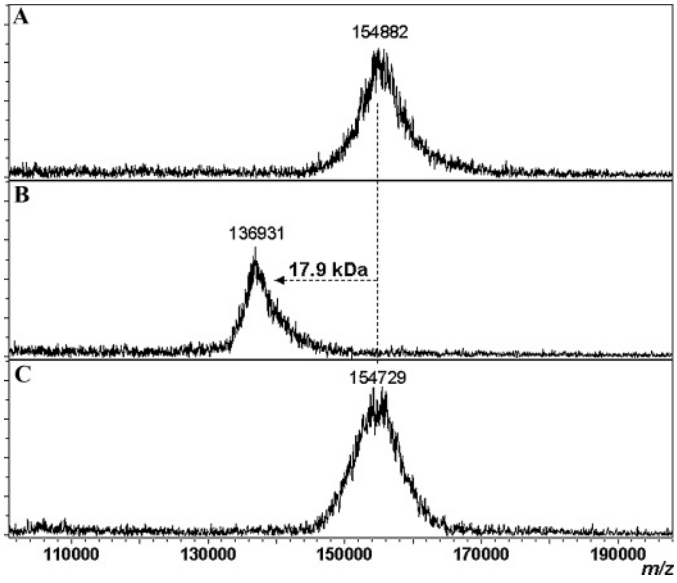


Fig. 3. Deglycosylation of CFH studied by MALDI-TOFMS. (**A**) Native CFH. (**B**) PNGase F-treated CFH. (**C**) Endo-H-treated CFH.

Peptide mapping of CFH

To determine oligosaccharide heterogeneity and the glycosylation sites, reduced and alkylated CFH was enzymatically digested into peptides and glycopeptides. The amino acid sequence of CFH is shown in Figure 1, and tryptic glycopeptides are shown in Table I, which summarizes the theoretical masses of the different tryptic peptides bearing the putative glycosylation sites, along with the masses of the major glycoforms measured by ESIMS and MALDI-TOFMS. Subtraction of the calculated mass of the tryptic peptides from the experimental molecular mass of the glycopeptides gave data on oligosaccharide residues. For example, the major glycoform mass was found to be ∼2204 Da, which corresponds to an A2S2 structure. The masses of the corresponding enzymatically deglycosylated peptides measured by MALDI-TOFMS are also given in

Table I. List of glycopeptides identified from CFH tryptic digest

Glycosylation site	Glycopeptide	$M_{Th.}$Mono.	Maj. Glycoform		$M_{exp.}$MALDI PNGase F	Mexp-Mth
			$M_{exp.}$ ESI	$M_{exp.}$MALDI		
Asn_{199}	194-SPDVI**NGS**PISQK-206	1340.7	1340.7	1340.7	ND	0
Asn_{511}	510-L**NDT**LDYECHDGYESNTGST TGSIVCGYNGWSDLPICYER-549	4617.9	6822.5	6825.7[a]	4619.1	2204.6
Asn_{700}	653-IQCVDGEWTTLPVCIVEEST CGDIPELEHGWAQLSSPPYY YGDSVEF**NCS**ESFTMIGHR-711	6856.5[a]	9061.2[a]	9062.5[a]	6857.5[a]	2204.7
Asn_{784}, Asn_{804}	779-WDPEV**NCS**MAQIQLCPPPPQ IPNSH**NMT**TTLNYR-812	4008.8	ND	8424.4[a]	4010.8	4415.6
Asn_{784}[b]	779-WDPEV**NCS**MAQ-789	1335.5	3540.3	3539.9	ND	2204.8
Asn_{804}[b]	790-IQLCPPPPQIPNSH**NMT**TTL-809	2258.1	4462.8	4464.8[a]	ND	2204.7
Asn_{864}	850-IPCSQPPQIEHGTI**NSS**R-867	2020.0	4224.8	4227.2[a]	2021.0	2204.8
Asn_{893}	889-ISEE**NET**TCYMGK-901	1560.6	3765.6	3765.3	1561.6	2205.0
Asn_{1011}	1006-MDGAS**NVT**CINSR-1018	1423.6	3628.5	3628.3	1424.6	2204.9
Asn_{1077}	1061-SPYEMFGDEEVMCLNG**NWT**EPPQCK-1085	3017.2	5222.1	5225.3[a]	3018.3	2204.9

[a]Average mass.
[b]Peptides resulting from pepsin digestion of (779–812) tryptic peptide.

Table I. Mass differences observed between theoretical peptides and PNGase F-treated glycopeptides indicate the number of glycosylation sites per glycopeptide, since the PNGase F-catalysed conversion of Asn to Asp generates a +1-Da mass shift. For example, the 2-Da mass difference observed for the (Trp_{779}-Arg_{812}) peptide suggests the presence of two glycosylation sites for this specific peptide.

If we consider the unmodified peptides, 71% of the amino acid sequence was covered by liquid chromatography (LC)-ESIMS/MS using trypsin (identified amino acids are underlined in Figure 1). This percentage increased to ∼77% when Asp-N was used. One peptide containing the potential N-glycosylation site Asn_{199} was detected, whereas peptides containing the other N-glycosylation sites were not detected. Thus, Asn_{511}, Asn_{700}, Asn_{784}, Asn_{804}, Asn_{864}, Asn_{893}, Asn_{1011} and Asn_{1077} might be glycosylated. When we consider the different glycopeptides, CFH sequence coverage increases to ∼91%.

Non-occupied Asn_{199} glycosylation site

Peptides containing unglycosylated Asn_{199} were identified in both trypsin and Asp-N digests. A mass of 1340.7 Da was obtained for the tryptic peptide, in agreement with the theoretical value (Table I). The MS/MS spectrum of the doubly charged ion at *m/z* 671.4 of this specific tryptic peptide is shown in Figure 4B. This spectrum could be interpreted via a series of b and y ions to give a high-confidence sequence for the peptide 194-SPDVI*N*GSPISQK-206. Based on mass analysis, we also observed two chromatographically separated peptides showing +1-Da mass difference compared to the peptide 194-SPDVI*N*GSPISQK-206. This observation is consistent with peptide deamidation. Figure 4A shows the extracted ion chromatograms of the doubly charged ions at *m/z* 671.9 corresponding to deamidated peptides. In addition to the peak originating from the isotopic contribution of the native peptide at 57.46 min, the extracted ion chromatogram of *m/z* 671.9 shows two distinct peaks at 56.38 and 59.12 min (Figure 4A), which potentially correspond to isoAsp- and Asp-containing peptides. MS/MS analysis confirmed the deamidated nature of these two species and the deamidation of Asn_{199} but did not enable distinction of isoAsp- and Asp-containing peptides. It is known that such distinction cannot be easily achieved by traditional collisional activation MS/MS methodologies (Cournoyer et al. 2006). Figure 4C displays the MS/MS spectrum of the isoAsp-containing peptide, which is very similar to one of the Asp-containing peptides (data not shown). Differentiation between isoAsp- and Asp-containing peptides was based on the elution times from reverse-phase HPLC, as isoAsp peptides elute earlier than their Asp isomers and Asn peptides (Chelius et al. 2005). Assuming similar ionization efficiencies for isoAsp- and Asp-peptides, an isoAsp/Asp ratio of ∼2.9 can be calculated by considering the corresponding peak areas (Figure 4A). This value is in good agreement with the commonly reported value of ∼3 (Geiger and Clarke 1987). Deamidation at this site was further confirmed by using data from Asp-N digestion (data not shown).

Under these analytical conditions, no glycosylated variants of this peptide were observed. Thus, we can conclude that Asn_{199} in the sequence NGS*P* is completely unoccupied (Mellquist et al. 1998).

Identification of glycopeptides by LC-ESIMS/MS

LC-ESIMS/MS was first used as a specific technique to identify glycopeptides by monitoring carbohydrate fragment ions (Huddleston et al. 1993). The MS/MS spectra of the different tryptic glycopeptides from CFH (Table I) show a very characteristic pattern (Figure 5) with intense oxonium ions at *m/z* 204 (HexNAc), *m/z* 366 (HexHexNAc), *m/z* 186 (HexNAc-H_2O), *m/z* 168 (HexNAc-$2H_2O$), *m/z* 138 (HexNAc-H_2O-CH_2O), *m/z* 274 (Neu5Ac-H_2O) and *m/z* 292 (Neu5Ac). When CFH is digested with trypsin, Asn_{784} and Asn_{804} are on the same peptide (Trp_{779}-Arg_{812}). To solve this problem and to individually characterize these glycosylation sites, the HPLC fraction containing this particular peptide was subsequently digested with pepsin to generate two distinct glycopeptides (Trp_{779}-Gln_{789}) and (Ile_{790}-Leu_{809}) containing one glycosylation site. Each peptide was reanalysed by LC-ESIMS/MS (Figure 5C and D). The eight spectra shown in Figure 5 were obtained from the major glycoform observed for each glycopeptide and are very similar to each other. Oligosaccharide composition was deduced from the molecular weight calculated from the observed masses of the

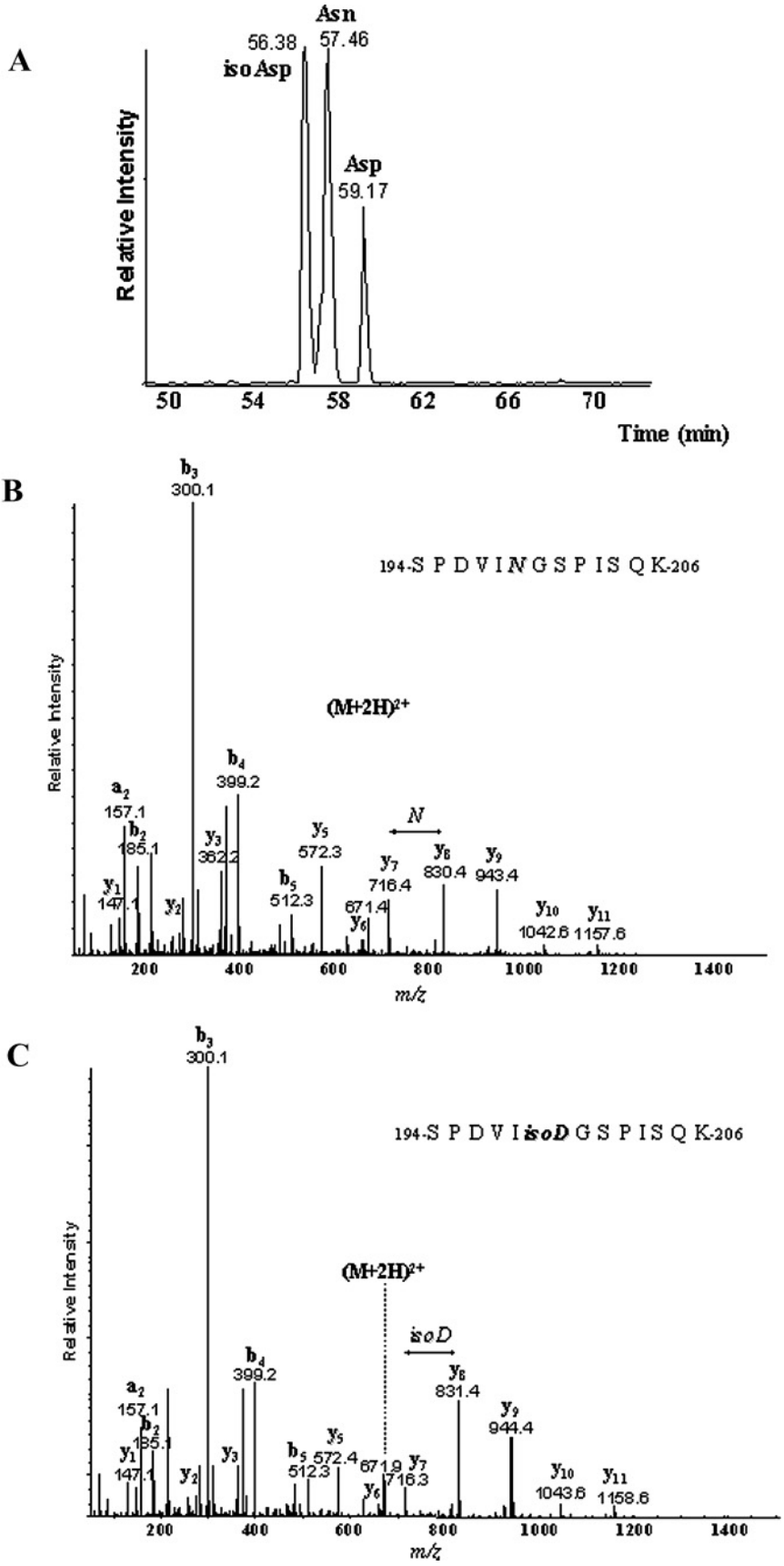


Fig. 4. (**A**) Extracted ion chromatogram of doubly charged ion at *m/z* 671.9, and MS/MS spectra of doubly charged ions at (**B**) *m/z* 671.4 and (**C**) *m/z* 671.9 corresponding to (Ser_{194}-Lys_{206}) tryptic peptides bearing Asn and isoAsp residues at position 199, respectively.

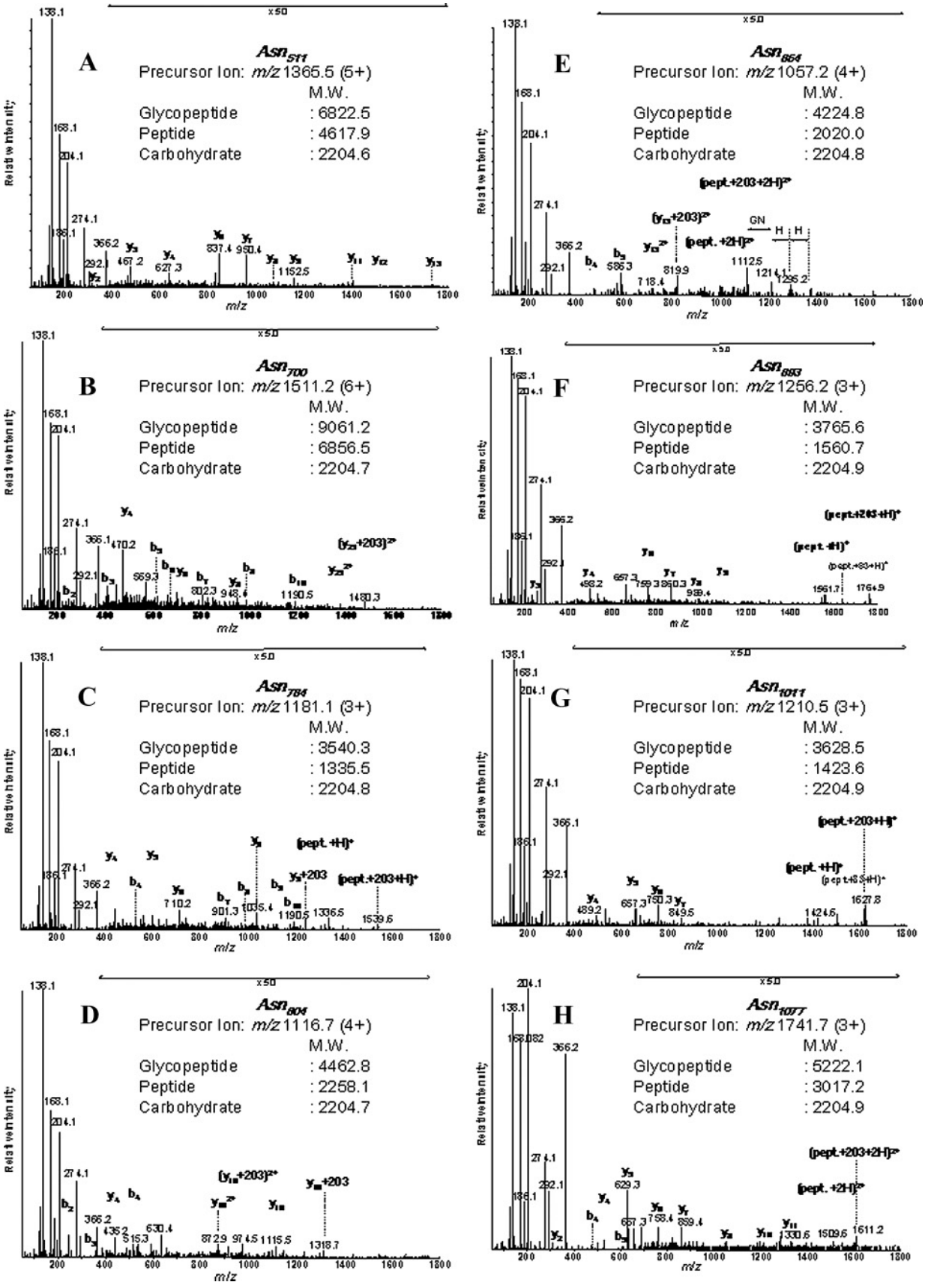


Fig. 5. MS/MS spectra of the eight different glycopeptides. Glycopeptide masses were calculated from the *m/z* value of the precursor ion and its corresponding charge state.

glycopeptide and the peptide. For example, Figure 5F shows the MS/MS spectrum of the triply charged ion at *m/z* 1256.2. In addition to the above-cited oxonium ions, several fragment ions of the y-series from peptide 889-ISEENETTCYMGK-901 were observed. The molecular ions of the peptide (*m/z* 1561.7) and peptide + GlcNAc (*m/z* 1764.9) were also detected. The molecular weight of the carbohydrate residue, 2204.9, was calculated from the observed molecular weight of the peptide (1560.7) and the calculated molecular weight of the glycopeptide (3765.6). Thus, an A2S2 carbohydrate composition was deduced. Table I summarizes the observed masses of the different glycopeptides.

As can be observed from Figure 5, the different glycopeptides carry the same major oligosaccharide structure whose calculated residue mass is ~2204.8 Da corresponding to a diantennary disialylated glycan (A2S2). These data are in good agreement with those generated by HPCE-LIF; thus, this type of glycan is the major structure and comprises 67% of the total glycan pool.

It should be noted that under conventional LC-ESIMS/MS fragmentation conditions, the most intense ions result from sugar losses. This yields information about the nature of the modification but less regarding peptide sequence or location of the modification (Mann and Jensen 2003). For instance, the MS/MS spectra in Figure 5, although giving enough b or y fragment ions to confirm the peptide sequences, do not enable the glycosylation site to be precisely identified. A deglycosylation step could be added to overcome this difficulty and facilitate the characterization of the peptides of interest. With this objective, glycopeptide containing HPLC fractions were analysed by MALDI-TOFMS before and after deglycosylation to further confirm peptide sequences and localize glycosylation sites. Edman sequencing was also performed to provide additional confirmation.

Identification of glycosylation sites

HPLC fractions containing glycopeptides were analysed by MALDI-TOFMS before and after deglycosylation with PNGase F, thus further confirming the oligosaccharide structures and the number of glycosylation sites on each individual peptide. These data are summarized in Table I, and Figure 6 depicts two specific examples of deglycosylation experiments. For instance, the glycopeptide (Ile_{850}-Arg_{867}) shows a major peak at *m/z* 4228.2, along with other peaks at *m/z* 3937.4 and *m/z* 3646.1 (average values, Figure 6A), coherent with A2S2, A2S1 and G2 glycoforms, respectively. It should be noted that A2S1 and G2 structures are, to some extent, artefactually generated by fragmentation of the A2S2 glycan during MALDI analysis (loss of sialic acid residues, i.e., 291 Da). Upon PNGase F treatment, the peak at *m/z* 4228.2 shows a mass decrease of ~2206 Da (Figure 6A), which is coherent with the loss of an A2S2 structure. Under these conditions, an intense ion can be observed at *m/z* 2022.0, which corresponds to the deglycosylated (Ile_{850}-Arg_{867}) peptide characterized by a PNGase F-catalysed conversion of Asn_{864} to Asp, which generates a monoisotopic mass shift of +0.9840 Da. Table I summarizes the masses observed before and after deglycosylation for the different peptides. Deglycosylation generates peptides that can be further analysed by MALDI-TOF/TOF experiments to confirm peptide sequences and localize glycosylation sites. For example, Figure 6B represents the results obtained for the peptide (Ile_{850}-Arg_{867}). This MS/MS spectrum shows intense fragment ions both from the b- and the y-series, which confirms the peptide sequence, and allows a precise localization of the glycosylation site.

This strategy was successfully applied to the glycopeptides containing Asn_{864}, Asn_{893} and Asn_{1011}. For the other glycopeptides, this strategy was not successful since deglycosylated peptides were too large to be sequenced by MALDI-TOF/TOF. To overcome this problem, Asp-N digestion was used to generate smaller sized peptides by cleaving at the N-terminal side of PNGase F-generated Asp residues. Thus, newly formed N-terminal Asp residues indicate N-glycosylation sites. This is exemplified in Figure 6C and D for the (Ile_{653}-Arg_{711}) peptide at *m/z* 9063.5, which is modified by an A2S2 glycan. Using this approach, we were able to identify the Asn_{700}, Asn_{785}, Asn_{904} and Asn_{1077} glycosylation sites. The glycosylation pattern of Asn_{511} was assessed as described, and the glycosylation site was confirmed by Edman sequencing and by working on glycopeptides coming from Asp-N digestion (data not shown).

Heterogeneity of oligosaccharides at each glycosylation site

The heterogeneity of carbohydrate structures was determined by LC-ESIMS as described above and their relative proportions estimated by working on deconvoluted mass spectra. Such an approach, involving glycopeptide analysis, has recently been demonstrated to be accurate and robust for oligosaccharide quantitation (Wada et al. 2007). Figure 7 represents the results obtained for the different glycopeptides. Irrespective of the glycosylation site, the A2S2 structure always constituted the major one. Other complex diantennary structures carrying none or one sialic acid could also be seen. Peptides located at the N-terminal also bore significant amounts of triantennary trisialylated glycans. Low levels of fucosylation were observed for these different structures. The relative intensity of the identified glycopeptides can be regarded as a semi-quantitative measure for the site distribution of glycosylation. These site-specific distributions of glycans were further confirmed by working on glycopeptides obtained by Asp-N digestion.

Discussion

Human CFH is a plasma protein that plays an essential role in the regulation of the alternative pathway of the complement system. Factor H can act as a cofactor for the plasma protease, factor I, to cleave C3b to its inactivated form iC3b. Furthermore, factor H can facilitate C3bBb dissociation and inhibit C5 convertase by competing with C5 for C3b binding. CFH's interaction with C3b is mediated by three different binding sites identified within SCRs 1–4, SCRs 6–10 and on SRCs 16–20 of CFH (Jokiranta et al. 2000; Aslam and Perkins 2001). The complement regulatory functions of CFH are modulated by GAGs with three heparin-binding sites located in SCR 7, SCR 13 and SCR 20 (Rodriguez de Cordoba et al. 2004).

Factor H is a 155-kDa glycoprotein containing nine potential N-glycosylation sites. Although the *N*-glycans linked to factor H have been studied (Ritchie et al. 2002), only a few carbohydrate structures have been reported and a site-specific characterization of these oligosaccharides has not been described. The aim of this study was therefore to provide an extensive site-by-site characterization of CFH's N-glycosylation profile.

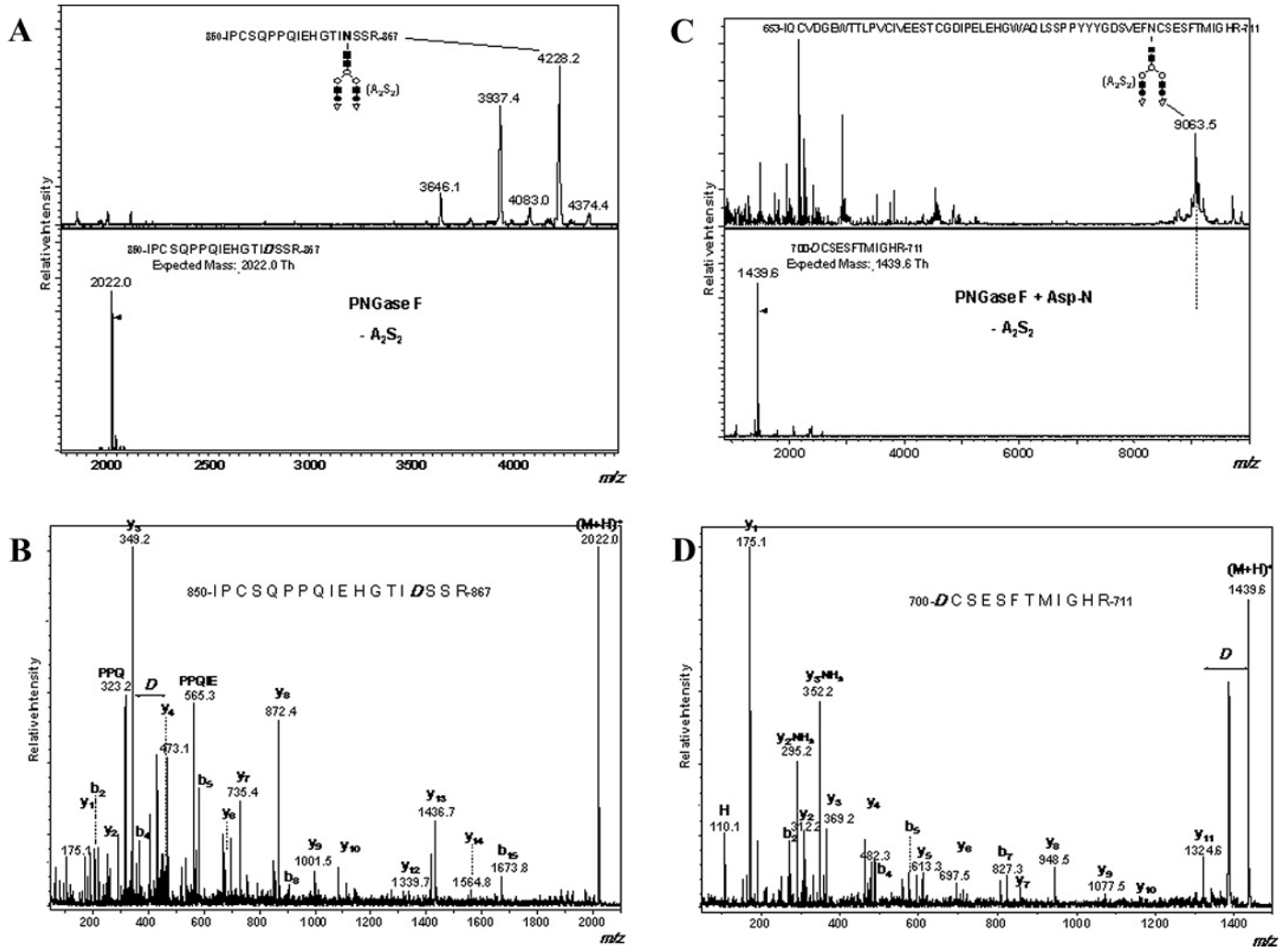


Fig. 6. Characterization of glycopeptides containing Asn_{864} and Asn_{700}. (**A**) and (**C**) MALDI-TOF mass spectra obtained before and after deglycosylation. (**B**) and (**D**) MALDI-TOF/TOF mass spectra of corresponding deglycosylated peptides. Mass spectrum in (C) was obtained on the glycopeptide containing Asn_{700} after deglycosylation with PNGase F and subsequent digestion with Asp-N.

In this study, the molecular mass of this protein was measured by MALDI-TOFMS at 154.8 kDa, which is in agreement with ultracentrifugation data reported by Aslam and Perkins (2001). After PNGase F treatment, a 17.9-kDa mass decrease (11.5% of total mass) was observed, indicating that N-glycosylation is the major post-translational modification of human factor H. This mass difference is consistent with CFH glycosylation by diantennary disialylated glycans of Mr 2204 Da on eight out of the nine potential sites. Factor H was not found to be sensitive to Endo H deglycosylation, indicating the absence of hybrid and oligomannose structures. We reported a quantitative glycosylation analysis of CFH using capillary electrophoresis. CFH predominantly contains complex, diantennary disialylated, non-fucosylated glycans (67%), without bisecting GlcNAc and minor disialylated, fucosylated and monosialylated non-fucosylated oligosaccharides (9%). Triantennary trisialylated fucosylated and non-fucosylated structures have also been detected. These account for approximately 8% of the total glycan pool. This is in agreement with data previously reported (Qiu and Regnier 2005a,b). The other complement regulatory factors, factor I and C4b binding protein, also presented similar oligosaccharide patterns with complex diantennary disialylated glycans, not bisecting GlcNAc and low levels of fucosylation (Ritchie et al. 2002).

In the current study, a complete characterization of the nine N-glycosylation sites was conducted, from the tryptic digest, using MALDI-TOFMS/MS and LC-ESI MS/MS. The amino acid sequence of CFH was covered and we have shown that among the nine potential N-glycosylation sites, only Asn_{199} was not occupied, while Asn_{511}, Asn_{700}, Asn_{784}, Asn_{804}, Asn_{864}, Asn_{893}, Asn_{1011} and Asn_{1077} were glycosylated. These results are in agreement with previously published data (Malhotra et al. 1999; Anderson and Hunter 2006). However, the absence of glycosylation for Asn_{199} remains controversial. Glycosylation sites have been identified using lectin affinity and deglycosylation with PNGase F generating an Asp_{199} residue (Wang et al. 2006). Asn-Gly sequences have been previously reported as particularly sensitive to deamidation (Krokhin et al. 2006), thus potentially explaining why the peptide (Ser_{194}-Lys_{206}) was also observed under its deamidated form. The presence of this deamidated form also confirms that this potential glycosylation site is not occupied. The ratio isoAsp/Asp was calculated at 2.9,

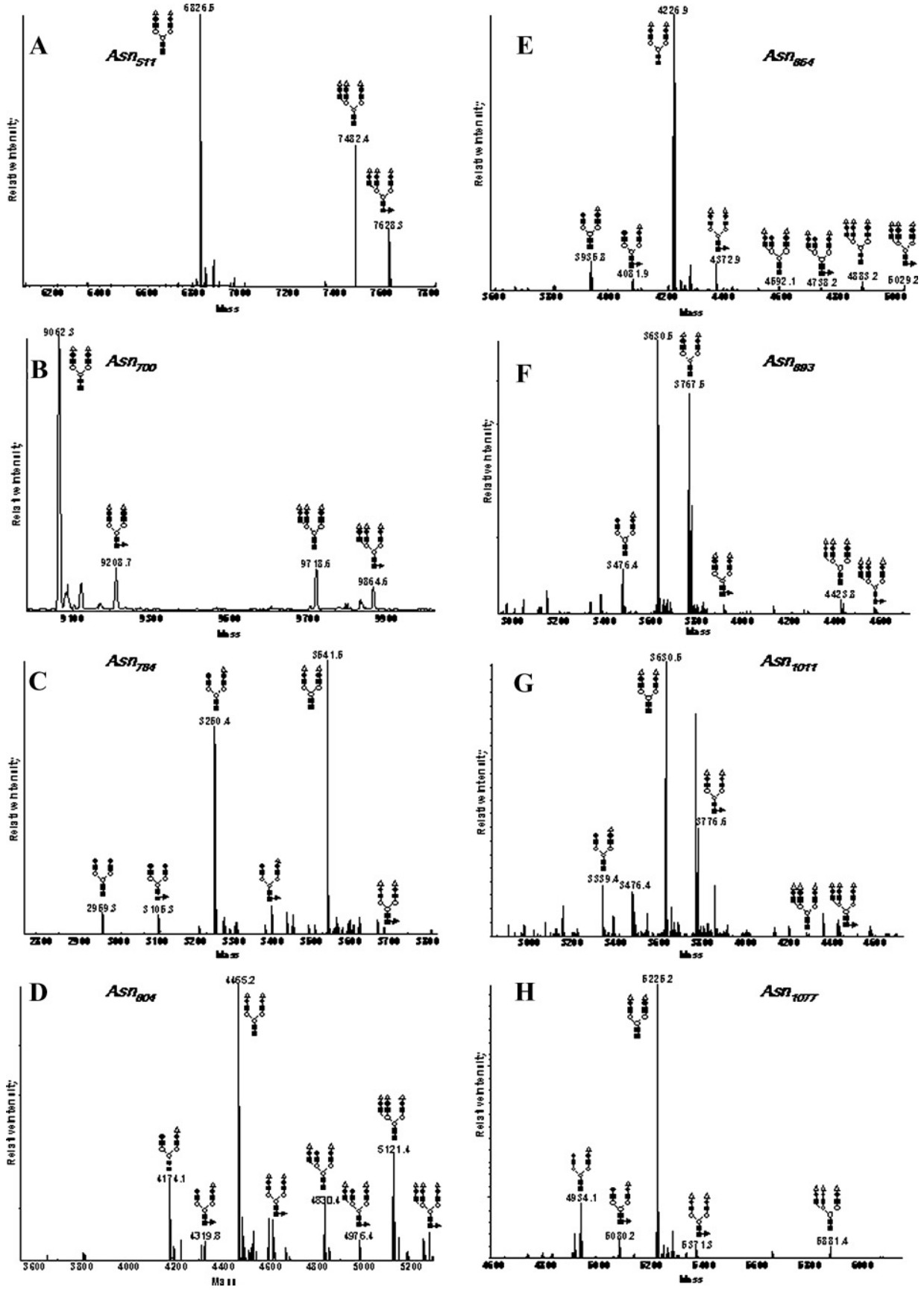


Fig. 7. Deconvoluted ESI mass spectra of the eight different glycopeptides. Masses are average masses. Some small peaks corresponding to trace amounts of glycans were observed but are not labelled because their corresponding structures were not fully elucidated.

which is in good agreement with the commonly reported value of ~3 (Geiger and Clarke 1987). This kind of peptide modification, i.e., ~70–80% conversion of Asn to Asp or isoAsp residues, can be observed under these experimental conditions. This artefactually generated deamidated peptide can be easily confounded with the one obtained by deglycosylation with PNGase F of a corresponding glycopeptide. Therefore, this underlines why large-scale glycoproteomics studies should preferably involve glycopeptide selection rather than glycoprotein selection prior to enzymatic deglycosylation, to avoid misleading results. This potentially explains some of the discrepancies regarding the occupancy of this particular glycosylation site.

In a large-scale glycoproteomics study involving the selective isolation and identification of glycopeptides from human plasma, a glycosylated form of this peptide was found but attributed to factor H-like protein 1 (FHL-1, isoform 2 of factor H) and not to CFH itself (Zhang et al. 2007). This may explain why some large-scale glycoproteomics studies reported the glycosylation of Asn_{199}, in contrast to studies dealing with purified CFH. We did not find any study reporting the glycosylation of FHL-1 in the literature, to further confirm the present assumption.

Several reasons for potential N-glycosylation sites not being occupied have been described such as the nature of the X and Y residues of the N-X-S/T-Y consensus sequence. Proline in the X or Y position is highly unfavourable for glycosylation (Mellquist et al. 1998). This can explain the absence of glycosylation at Asn_{199} of the NGS*P* sequence of CFH.

MS analysis allowed consensus sequence verification of the glycosylation sites and site-specific identification of the different oligosaccharide structures. Asn_{511}, Asn_{700}, Asn_{784}, Asn_{804}, Asn_{864}, Asn_{893}, Asn_{1011} and Asn_{1077} were glycosylated essentially by diantennary disialylated structures with a relative distribution varying between 45% for Asn_{804} and 75% for Asn_{864}. Diantennary monosialylated glycans and triantennary trisialylated fucosylated and non-fucosylated structures have also been identified. Asn_{511} is located in the SCR9 of the C3b-binding site, while Asn_{1011} and Asn_{1077} are respectively in SCR17 and SCR18 of the C3b-binding site of CFH. The interaction of CFH with C3b is predominantly ionic in nature and so the presence of charged sialylated glycans may reinforce the protein–protein interaction. CFH is a ligand for a glycoprotein involved in the inflammation process, L-selectin, and CFH sialylation is implicated in this binding (Malhotra et al. 1999).

Significant differences in the relative distribution of oligosaccharides between the various glycosylation sites were found. Oligosaccharide maps of the N-glycosylation site localized at the N-terminal were clearly different from those at the C-terminal. At Asn_{511}, high amounts of triantennary trisialylated forms were found (40% when considering the fucosylated and non-fucosylated glycans) and we noticed the absence of diantennary monosialylated structures. By contrast, at Asn_{1077}, low amounts of triantennary trisialylated structures were found (3%) while the diantennary monosialylated forms represented 20% of the glycans. The difference between the glycosylation patterns of the N-glycosylation sites of CFH could be related to the position in the peptide chain. It has been suggested that when the distance of the glycosylation site from the C-terminal increases, the complexity of the oligosaccharides increases (Rudd and Dwek 1997).

Interestingly, among the three heparin-binding sites, SCR7 and SCR20 did not contain any glycosylation site while SCR13 contained Asn_{784}. This N-glycosylation site was 50% modified by monosialylated structures corresponding to the lower level of sialylation. We suggest that the local three-dimensional conformation of the protein around this site affects the accessibility of glycosyltransferases to the oligosaccharide, modulating the glycan structure of the secreted glycoprotein. This finding would suggest that CFH's three-dimensional structure is not as extended as it could be (Aslam and Perkins 2001), but is rather compact (Oppermann et al. 2006) leading to poor accessibility to the SCR13 glycosylation site.

Despite that SCR 12 and SCR 13 contain the same NCS glycosylation consensus sequence, their glycosylation patterns are different. Indeed, less mature oligosaccharide structures are found at Asn_{784} (SCR 13). This could be linked to its unique position at the C-terminus (linker region) of the SCR.

Data from the literature suggest that the CFH N-linked glycans may have structural rather than functional roles in the interactions with the natural biological partners, by regulating the areas of protein surface exposed for protein–protein interactions. The site-specific characterization of the N-linked glycans of CFH described in this paper would facilitate targeted partial deglycosylation approaches for subsequent functional analysis, to further obtain more insight into the role of N-linked glycans in the mechanism of action of CFH.

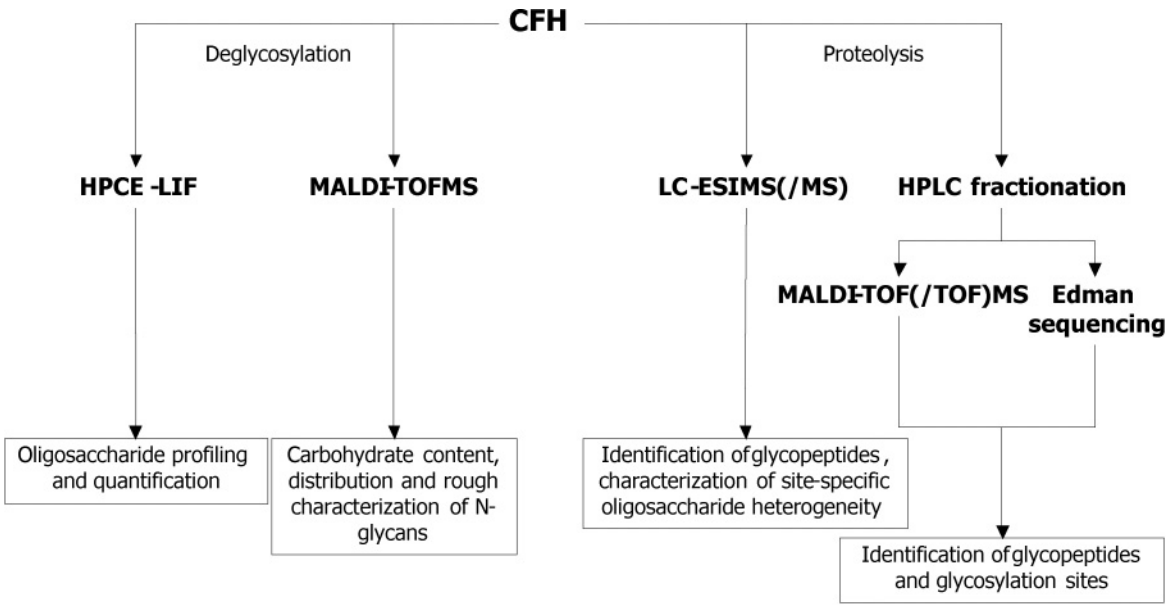
Materials and methods

Analytical approach

The general approach used is shown in Scheme 1. First, qualitative and quantitative profiling of the *N*-glycan structures was obtained by HPCE-LIF. Then, MALDI-TOFMS combined with selective enzymatic deglycosylation of CFH was used to provide further information on the distribution and structure of oligosaccharides. In parallel, CFH was reduced, carboxymethylated and then proteolytically digested with enzymes of different specificity (trypsin and Asp-N). Enzymatic digests were then both directly analysed by LC-ESIMS(/MS) using a hybrid quadrupole/time-of-flight (Q-TOF) instrument, and fractionated by HPLC for subsequent MALDI-TOFMS analysis and Edman sequencing.

Materials

Unless otherwise specified, all reagents were of analytical grade. The MALDI matrices α-cyano-4-hydroxycinnamic acid (HCCA) and 2,5-dihydroxybenzoic acid (DHB) were purchased from Laser BioLabs (Sophia Antipolis, France) and 2,4,6-trihydroxyacetophenone (THAP) was from Fluka (Saint Quentin Fallavier, France). Dithiothreitol (DTT) and iodoacetamide were obtained from Sigma–Aldrich (Saint Quentin Fallavier, France). Trypsin, Asp-N and pepsin were purchased from Roche (Mannheim, Germany). Peptide-*N*-glycosidase F (PNGase F) and Endo H were from Prozyme (San Leandro, CA) and Sigma–Aldrich, respectively. High-purity 1-aminopyrene-3,6,8-trisulphonate (APTS) was purchased from Beckman Coulter (Fullerton, CA). CFH was purified in-house from a pool of human plasma, and has a purity of ~94% as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Functional characterization indicated that the purified CFH had cofactor I (DiScipio 1994) and



Scheme 1. Analytical methodology.

decay-accelerating (Hourcade et al. 1999) activities similar to those of human plasma (data not shown).

Deglycosylation of CFH for MALDI-TOFMS analysis

CFH was enzymatically digested using PNGase F and Endo H in organic-aqueous solvent systems (Fenaille et al. 2007). Briefly, 1 μL of a CFH aqueous solution (i.e., 2 μg) was mixed with 6 μL of acetonitrile/water (75:25, v/v) and 0.5 μL of a PNGase F (i.e., 1.25 mU) or Endo H solution (i.e., 2.5 mU). The resulting mixture was incubated for 1 h at 37°C. Deglycosylated CFH was further analysed by MALDI-TOFMS to estimate the contribution of N-glycosylation on protein mass (see below).

Deglycosylation of CFH and oligosaccharide derivatization for quantification by HPCE-LIF

CFH deglycosylation was performed according to manufacturer's instructions using a Prozyme deglycosylation kit (San Leandro, CA). Briefly, 100 μg of lyophilized CFH were resuspended in 46.5 μL of 20 mM sodium phosphate buffer pH 7.5. CFH was further denatured by addition of SDS and β-mercaptoethanol (2 μL of a 2% SDS, 1 M β-mercaptoethanol solution). After a 15-min incubation at room temperature (RT), 0.5 μL of a 15% Nonidet NP-40 solution was added prior to the addition of 1 μL of PNGase F solution (2.5 mU/μL). Deglycosylation was performed overnight at 37°C. Proteins were then precipitated by 150 μL of ice-cold ethanol. Supernatant containing released glycans was vacuum-dried and solubilized in 2 μL of APTS solution (5 mg in 48 μL of 15% acetic acid) and 2 μL of 1 M sodium cyanoborohydride solution in THF (Sigma-Aldrich). The mixture was then incubated overnight at 37°C. Finally, the solution was diluted by adding 46 μL of water prior to analysis by HPCE-LIF.

Proteolytic digestion of CFH

Approximately 100 μg of lyophilized CFH were dissolved in 50 μL of 8 M urea, 0.4 M ammonium bicarbonate solution pH 8.0. Disulphide bridge reduction was accomplished by adding 10 μL of a 100 mM DTT solution in water and incubation for 45 min at 56°C. The solution was allowed to cool at RT, before adding 10 μL of a 200 mM iodoacetamide solution in water. The solution was then incubated at RT for 30 min in the dark. After an eight-fold dilution in water, digestion with trypsin (1:25, w/w) or Asp-N (1:100, w/w) was performed by overnight incubation at 37°C.

Fractionation of CFH digests by LC

The peptides obtained after trypsin or Asp-N digestion were fractionated using a Uptisphere C18 column (250 × 3 mm i.d., 5 μm, Interchim, Montluçon, France) mounted on an Akta purifier FPLC system (GE Healthcare, Saclay, France). Detection was performed at 214 and 280 nm, and the flow rate was set to 0.5 mL/min. Solvent A was 0.12% TFA and solvent B was acetonitrile containing 0.1% TFA. The gradient used was 0% B for 10 min, 0–5% B in 2 min, 5–35% B in 120 min, 35–50% B in 15 min, and finally, 50–90% B in 20 min. In all, 700-μL fractions were collected in the range 15–120 min and evaporated to dryness. Fractions were then reconstituted in 10 μL of water prior to MALDI-TOFMS analysis or Edman sequencing.

Enzymatic digestions of HPLC fractionated peptides

CFH digestion with trypsin or Asp-N generates a peptide that still contains two potential glycosylation sites: Asn_{784} and Asn_{804}. To individually characterize these glycosylation sites, the HPLC fractions containing the tryptic peptide (Trp_{779}-Arg_{812}) were subsequently digested with pepsin to generate two distinct glycopeptides (Trp_{779}-Gln_{789}) and (Ile_{790}-Leu_{809}). For this purpose, 3 μL of the three fractions containing the glycopeptide of interest were mixed together with 2 μL of a pepsin solution (0.2 mg/mL in 0.02 N HCl) and the mixture was incubated for 2 h at 37°C.

On-target deglycosylation of the glycopeptides was performed (Colangelo and Orlando 2001). Briefly, 0.5 μL of the

LC fraction dried and resuspended in water, 0.5 μL of PNGase F solution, and 1.5 μL of water were thoroughly mixed on the MALDI target. The reaction was allowed to proceed for 15 min at RT, and stopped by adding 0.5 μL of the matrix solution. In order to fully identify N-glycosylation sites, PNGase F and Asp-N digestions were consecutively performed on-target. Indeed, PNGase F treatment leads to the conversion of N-glycosylated asparagine (Asn) to aspartic acid (Asp) residues, thus generating an additional Asp-N cleavage site. Therefore, newly formed N-terminal Asp residues indicate N-glycosylation sites. This was achieved by first performing on-target deglycosylation as described above, and then adding 0.5 μL of Asp-N solution (40 μg/mL) and another 1.5 μL of water to the mixture. Digestion was performed for 30 min at RT, water was added as needed to prevent the sample from drying out. Matrix was added to quench the reaction.

MALDI-TOFMS analyses

Analyses were carried out on an Autoflex II mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with a nitrogen laser. Analyses were carried out using different matrix solutions: (i) HCCA, 0.3 mg/mL in ethanol/acetone (2:1, v/v); (ii) DHB, 20 mg/mL in acetonitrile; (iii) THAP, 10 mg/mL in acetonitrile/water (1:1, v/v) containing 0.1% TFA. HCCA and DHB matrices were used for the analysis of proteolytic peptides and glycopeptides, respectively; THAP was used for the analysis of native and deglycosylated proteins (Fenaille et al. 2007). For each sample, 0.5 μL of analyte solution and 0.5 μL of matrix solution were deposited and thoroughly mixed on the target. For protein analysis, ions were analysed in the linear mode after acceleration at 20 kV, with an extraction delay of 500 ns. Laser energies were 30–45% depending on the protein. Peptide analysis was performed in the reflectron mode with an acceleration of 19 kV and a delayed extraction time of 110 ns. Laser energy ranged from 20 to 30% depending on the peptide and matrix. External calibration was achieved by the use of standard mixtures, purchased from Bruker Daltonik GmbH. A total of 100–200 single spectra were collected to produce a MALDI mass spectrum.

LC-ESIMS/MS analysis of CFH digests

Reversed-phase chromatography was performed using an Agilent 1100 HPLC system (Palo Alto, CA). Separation was achieved using an Uptisphere C18 column (150 × 2 mm i.d.) at a flow rate of 250 μL/min and kept at 40°C. Buffer A was 0.1% formic acid, while buffer B was acetonitrile containing 0.1% formic acid. The gradient separation was 2–35% B over 110 min. Peptides eluted from the column were introduced on-line into an ESI-QqTOF instrument (QSTAR XL, Applied Biosystems, Toronto, Canada). The ionspray voltage was 5 kV. All LC-MS/MS analyses were performed using data-dependent switching between MS and MS/MS modes. MS cycles comprised a TOFMS survey scan with an *m/z* range of 300–1800 Th for 1 s, followed by two product ion scans with an *m/z* range of 50–1800 Th for 2 s each. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. After a given precursor was selected, dynamic exclusion was used for the next 60 s to prevent its subsequent reselection.

Oligosaccharide quantification by HPCE-LIF

APTS-labelled glycans were separated by HPCE-LIF. HPCE-LIF analyses were performed on a Beckman Proteome Lab PA800 capillary electrophoresis system equipped with an argon-ion laser with an excitation wavelength of 488 nm and an emission band-pass filter of 520 nm. Separations were carried out in a 50 cm × 50 μm i.d. N-CHO-coated capillary (Beckman Coulter) using 'gel buffer N' separation buffer (Beckman Coulter). The applied voltage was 25 kV and the temperature was set at 20°C. The structural assignments of CFH oligosaccharides were made by matching their HPCE retention times with those of well-characterized standard oligosaccharides of complex type, as commonly performed using such a technique. These structural assignments were further confirmed by performing specific desialylation and defucosylation experiments (data not shown).

Edman sequencing

N-terminal sequencing was performed on a Procise 491 protein sequencer (Applied Biosystems) using standard procedures.

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Conflict of interest statement

None declared.

Abbreviations

aHUS, atypical haemolytic uraemic syndrome; CFH, complement factor H; CFI, complement factor I; DTT, dithiothreitol; Endo H, endoglycosidase H; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PNGase F, peptide-N-glycosidase F; SCR, short consensus repeat; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; THAP, 2,4,6-trihydroxyacetophenone; TOF, time-of-flight.

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