REVIEW ARTICLE

Molecular structures of glycoprotein hormones and functions of their carbohydrate components

Anne STOCKELL HARTREE* and Alistair G. C. RENWICK†

*AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT, U.K., and †Department of Biochemistry, University of Auckland, Private Bag, Auckland, New Zealand

INTRODUCTION

The glycoprotein hormones discussed in this review belong to a family of homologous proteins which are secreted by the anterior pituitary gland of all vertebrates as well as by placental cells of primates and equids. The pituitary glycoprotein hormones include follitropin (follicle-stimulating hormone, FSH) and lutropin (luteinizing hormone, LH), essential regulatory elements of ovary and testis which are required for reproduction, and thyrotropin (thyroid-stimulating hormone, TSH), which is indispensable for the control of thyroid structure and function and ultimately metabolism. Placental cells of primates and equids secrete choriogonadotropin (chorionic gonadotrophin, CG), which also stimulates gonadal tissue. In addition there is aberrant production of portions of these proteins and/or complete hormone molecules by certain types of tumour cells.

Each hormone is transferred via the blood stream to its target tissue, and its biological activity is dependent upon an initial binding to a specific receptor on the surface of a target cell. This evokes a chain of events, via specific response pathways involving chemical 'messengers' (such as cyclic AMP and/or calcium), which culminates in the response typical of the hormone. In the female mammal, follitropin stimulates growth of ovarian follicles, whereas in the male it promotes spermatogenesis. Subsequent stages in the female reproductive cycle are dominated by lutropin, which stimulates ovulation of the follicle and its conversion to the corpus luteum which in turn secretes progesterone; in the male, lutropin stimulates the Leydig cells to secrete androgen. Human choriogonadotropin, which has been studied extensively, possesses the biological activity of lutropin in the main, but the choriogonadotropin produced by the pregnant mare (known as equine choriogonadotropin, PMSG or pregnant mare serum gonadotropin) exhibits appreciable follitropin activity in many non-equine mammals in addition to its lutropin activity. Thyrotropin in both sexes stimulates growth and secretion of

thyroid gland cells. The corresponding hormones of lower vertebrates often have biological activities that differ from those of mammals. In addition, there is evidence in some of the former that a single gonadotropic hormone replaces mammalian follitropin and lutropin [1].

A great deal of information is now available on the amino acid sequences of these hormones from various vertebrate species. Studies employing the use of specific antibodies, chemical or enzymic modification and site-directed mutagenesis have identified certain regions of these proteins that are important in the maintenance of structural integrity and in receptor binding and transduction of the hormonal response. The attached carbohydrate is apparently required for efficient formation of their specific folding patterns and for full expression of biological activity, and the structures of some of the individual carbohydrate side chains have recently been published. Much new information has appeared since publication of earlier reviews on structure–function relationships of these hormones [2,3] and on structure, synthesis and function of some of their carbohydrate moieties [4]. This Review attempts to summarize currently available structural information on the protein and carbohydrate components of these hormones as well as the functions thus far established for the latter.

MEASUREMENT OF HORMONE ACTIVITY

The earliest assays for these hormones measured biological responses in test animals *in vivo*. Mammalian lutropin activity can be measured by its effectiveness in increasing the weight of the ventral lobe of the prostate gland of hypophysectomized rats [5] or in depleting ovarian ascorbic acid in pseudopregnant intact female rats [6]. Follitropin assays are based on its effectiveness in increasing ovarian weight in immature intact female rats [7] or mice [8] pretreated with excess human choriogonadotropin.

In vitro bioassays for these activities, which are more sensitive

	10	20	30	40	50	60	70	80	90																																																																																							
Bovine	F	P	D	G	E	F	T	M	Q	G	C	P	E	C	K	L	K	N	K	Y	F	S	K	P	D	A	P	I	Y	Q	C	M	G	C	C	F	S	R	A	Y	P	T	P	A	R	S	K	K	T	M	L	V	P	K	N	I	T	S	E	A	T	C	C	V	A	K	A	F	T	K	A	T	V	M	G	N	V	R	V	E	N	H	T	E	C	H	C	S	T	C	Y	Y	H	K	S	
Human	A	P	D	V	Q	---	---	---	---	D	C	P	E	C	T	L	Q	E	N	P	F	F	S	Q	P	G	A	P	I	L	Q	C	M	G	C	C	F	S	R	A	Y	P	T	P	L	R	S	K	K	T	M	L	V	Q	K	N	V	T	S	E	A	T	C	C	V	A	K	S	Y	N	R	V	T	V	M	G	G	F	K	V	E	N	H	T	E	C	H	C	S	T	C	Y	Y	H	K	S
Equine	F	P	D	G	E	F	T	T	Q	D	C	P	E	C	K	L	R	E	N	K	Y	F	F	K	L	G	V	P	I	Y	Q	C	K	G	C	C	F	S	R	A	Y	P	T	P	A	R	S	R	K	T	M	L	V	P	K	N	I	T	S	E	A	T	C	C	V	A	K	A	F	I	R	V	T	V	M	G	N	I	K	L	E	N	H	T	E	C	Y	C	S	T	C	Y	H	K	I	
Salmon	Y	Q	N	S	D	M	T	N	V	G	C	E	C	K	L	K	N	K	V	F	S	N	P	G	A	P	V	Y	Q	C	T	G	C	C	F	S	R	A	Y	P	T	P	L	Q	S	K	K	A	M	L	V	P	K	N	I	T	S	E	A	T	C	C	V	A	K	E	G	E	R	V	V	-	D	N	I	K	L	N	H	T	E	C	W	C	N	T	C	Y	H	K	S					
Carp	Y	P	R	N	D	M	N	F	G	C	E	C	K	L	K	N	I	F	S	K	P	G	A	P	V	Y	Q	C	M	G	C	C	F	S	R	A	Y	P	T	P	L	R	S	K	K	T	M	L	V	P	K	N	I	T	S	E	A	T	C	C	V	A	K	E	V	K	R	V	L	V	-	N	D	V	K	L	N	H	T	E	C	H	C	S	T	C	Y	Y	H	K	S					

Fig. 1. Comparison of amino acid sequence of α -subunits from mammalian and fish sources

The numbering above the sequences corresponds to that of bovine α -subunit and gaps (–) have been introduced in the human sequence at a suggested four-codon gene deletion [30] and in the salmon and carp sequences to optimize similarity. The positions of N-glycosylation are indicated by asterisks. The residues shown in red are invariant in all reported mammalian sequences, including bovine [31,32], human [33,34], equine [35,36], rhesus monkey [37], ovine [38–40], porcine [41–43], mouse [44], rat [45], rabbit [46] and whale [47]. The sequences of chum salmon gonadotropin type I α -subunit [50,51] and carp type I α -subunit [52], with residues in red which are identical with those invariant in mammals, are included to illustrate the high degree of similarity in diverse vertebrate species. As discussed in the text, there is good evidence for disulphide bonds linking residue 11 with 35 and 14 with 36 [60], but there is still uncertainty concerning the postulated bonds linking residue 30 with 64, 63 with 91 and 86 with 88 [58, 59].

and convenient and less time-consuming than *in vivo* methods, are now widely used. Lutropin has been measured in assays based on its stimulation of testosterone production by mouse [9] or rat [10,11] testicular Leydig cells, by its stimulation of progesterone secretion in a mouse Leydig tumour cell line [12] and by its stimulation of cyclic AMP accumulation by mouse Leydig cell suspensions [13]. *In vitro* measurement of follitropin activity has been based on its stimulation of the conversion of 19-hydroxyandrostenedione to oestradiol by cultured rat Sertoli cells [14] or on its stimulation of oestrogen production from androstenedione by rat granulosa cells [15]. These and other bioassays for follitropin have been reviewed by Wang [16].

The biological activities of follitropin and lutropin in lower vertebrates are not necessarily the same as those of mammals. Many non-mammalian species possess two distinct pituitary gonadotropins, which have little or no activity in mammalian systems. However, mammalian lutropin and follitropin are often highly active in lower vertebrates, and bioassays which are relatively specific for each hormone have been developed in these species [1]. Follitropin can be assayed by its stimulation or maintenance of testicular activity in the lizard, and lutropin specifically stimulates ovulation of follicles from frog ovarian segments. Lower vertebrate gonadotropins have been classified as lutropin or follitropin according to their specificities in these assays [1].

Bioassays for thyrotropin have been reviewed by Bates & Condliffe [17]. A widely used *in vivo* procedure measures thyrotropin stimulation of secretion of radioactively labelled material by chicks [18], guinea pigs [19] or mice [20] following injection of radioactive iodine. *In vitro* methods have been established, which measure radioactivity released by cultured thyroid tissue from guinea pigs pretreated with methyl thiouracil and radioactive iodine [21] or from mice kept on a low-iodine diet and treated with radioactive iodine [22].

Immunological methods of assay, which measure binding of the hormone to specific antibodies, were first developed around 1960 and are now widely used, particularly to determine hormone concentrations in physiological fluids. Assays based on specific binding of hormone to its tissue receptor became available during the 1970s, and in both types of binding assay the competition between radioactively labelled hormone and test substance for specific binding to antibody or to receptor can be determined. Other types of immunoassay employ labelled antibodies which specifically bind the hormone being measured. However, all immunoassays depend on antibody reactions at specific antigenic sites and thus do not necessarily reflect biological activity as do the receptor assays.

SUBUNIT STRUCTURE

Each glycoprotein hormone consists of two subunits, designated α and β , which are single polypeptide chains with carbohydrate attached at one or more sites in each. Within the same species the α -subunits of each hormone have similar, if not identical, amino acid sequences, but the β -subunits are different and are the determinants of hormone specificity. In the intact hormone the subunits are held together by non-covalent bonds as they can be dissociated by treatment with urea, guanidine or dilute mineral acid. Isolated subunits possess little or no biological activity, but they can be recombined with restoration of hormonal function. When hybrid molecules are prepared by combining α - and β -subunits of different glycoprotein hormones from the same vertebrates species, each recombinant exhibits the biological activity of the hormone from which its β -subunit was derived.

Subunits from remotely related species can also associate to

form biologically active hybrids. For example, turtle lutropin subunits could recombine with their counterpart subunits derived from ovine lutropin, and these hybrid molecules were active in the frog ovary assay [23]. When subunits isolated from a fish gonadotropin were tested for hybridization with mammalian lutropin subunits, carp α -subunit did not recombine with ovine lutropin- β , but carp β -subunit did combine with ovine- or bovine- α to yield a hybrid hormone which had high activity in a frog spermiation assay [24,25].

Biosynthesis of each subunit is accomplished by the complex processes established for other glycoproteins. After the genetic information is transcribed and translated, via mRNA, certain post-translational modifications occur in the rough endoplasmic reticulum, including addition of high-mannose N-linked oligosaccharides and cleavage of the 'signal' or 'leader' sequence. There is evidence that association of α - and β -subunits begins in the rough endoplasmic reticulum [26–28]. The remaining stages of N-linked oligosaccharide synthesis, sulphation and all O-linked glycosylation are believed to occur in the Golgi apparatus, where glycosylation reactions are considerably more diverse than in the rough endoplasmic reticulum [29].

It has been apparent for some time that these hormones exhibit microheterogeneity due to minor chemical variations in their carbohydrate moieties as well as in their polypeptide chains. In some cases these differences are believed to result in changes in biological activity. However, these multiple forms of a given hormone or subunit are nearly always encoded by a single gene and their differences result from translational or post-translational events. The sources of microheterogeneity are discussed further in the sections on amino acid sequences of subunits and carbohydrate structures.

AMINO ACID SEQUENCES OF α -SUBUNITS

The amino acid sequences of α -subunits from nine mammalian [31–47], one amphibian [48] and one avian [49] species and from five types of fish [50–54] have been determined, either by direct sequencing of the polypeptide chain or by deduction from the nucleotide sequence of the gene or of the cDNA corresponding to the mRNA which codes for the protein. For some α -subunits both methods have been used, and when discrepancies occur it has been assumed that the deduced sequence is more likely to be correct. The sequences of three mammalian and two fish α -subunits are shown in Fig. 1, which illustrates the high degree of similarity found when sequences of this subunit from very diverse species are compared. Throughout this Review the numbering of α -subunit residues is that of bovine- α (Fig. 1).

In sequence studies of α -subunits, heterogeneity at the N-terminus has been reported with up to six amino acid residues missing in some cases, but this does not appear to affect adversely the biological activities of the hormones. No C-terminal heterogeneity has been reported, and this portion of the subunit appears to be important in the expression of biological activity. Enzymic removal of five residues at the C-terminus resulted in loss of biological activity of bovine lutropin [55] and of human choriogonadotropin [56], and site-directed mutagenesis of the penultimate Lys residue of the latter to Met or Glu (but not Arg) also led to loss of biological activity [57].

All α -subunits thus far investigated contain N-linked carbohydrate at Asn residues 56 and 82, and the mRNA coding for α -subunit in mammals also encodes a 24-residue pre-sequence. The similarity between species is greatest in the neighbourhood of the ten disulphide-bonded half-cystine residues (which are all conserved), and particularly in the regions comprising residues 28–67 and 81–92. Because of their high degree of similarity and the ease of formation of hybrid hormones with β -subunits of

different origin, α -subunits are assumed to have similar conformations and thus identical positions of disulphide bonds.

Partial reduction of bovine lutropin α -subunit [58] or human choriogonadotropin α -subunit [59] followed by carboxymethylation with radioactively labelled reagent led to assignments of disulphide bonds linking residue 11 with 35, 14 with 36, 32 with 64, 63 with 91 and 86 with 88. However, the latter three pairings are uncertain because of similarities in their degree of labelling. A single disulphide bond linking residues 11 and 35 in bovine lutropin- α could be reduced and alkylated without significantly altering the conformation of the subunit, which could still combine with β -subunit, but reduction of a second bond linking residues 14 and 36 resulted in loss of tertiary structure [60].

In addition to the α -subunit present in glycoprotein hormones, significant amounts of 'free α -subunit', which does not combine with β -subunit, have been found in bovine [61] and mouse [62] pituitary fractions and in human placental tissue [63–65]. This 'free α -subunit' differs from the combined form in its oligosaccharide structure, which is believed to prevent its combination with β -subunit. A site of O-linked glycosylation (at Thr-43) has been found in bovine 'free α -subunit' [61] but not in human placental 'free α -subunit', which appears to be more heavily glycosylated in its N-linked carbohydrate moieties than the subunit present in human choriogonadotropin [65,66].

No definite function for 'free α -subunit' has yet been found, although the human protein isolated from pregnancy urine was shown to stimulate the release of prolactin from cultured human decidual cells at concentrations similar to those found in serum during pregnancy [67].

AMINO ACID SEQUENCES OF β -SUBUNITS

Amino acid sequences of lutropin- β from nine mammals [47, 68–78], choriogonadotropin- β from three mammals [33,79–83], bullfrog lutropin- β [84], chicken lutropin- β [85], and the β -subunits of fish gonadotropin from four species [52,54,86–90] have been reported and found to be highly similar. The amino acid sequence of follitropin- β has been determined in six mammals [42,78,91–101] and thyrotropin- β in five mammals [31,43,102–113]. Each choriogonadotropin β -subunit sequence and that of equine lutropin possesses a C-terminal extension of 25–30 amino acids containing at least four O-linked carbohydrate moieties [33,79–83].

There are similarities between β -subunit sequences from different hormones [114], as illustrated in Fig. 2 where the human sequences are aligned so that the positions of their Cys residues coincide. In this Review the numbering of residues is that of human choriogonadotropin- β . Investigations of primary structure have demonstrated that heterogeneity, involving cleavage of up to five amino acid residues, can occur at the C-terminus of the peptide chain of all types of β -subunits, but only those of follitropin exhibit N-terminal heterogeneity. There is no evidence of any adverse effect of this type of heterogeneity on hormonal activity.

A pre-existing internal peptide bond cleavage has been reported in the region of β -subunit residues 44–49 in a proportion of human lutropin molecules in all preparations examined [120,121,124–127]. Similar nicks have been reported in the corresponding regions of choriogonadotropin [128,129] and follitropin [91], and there is evidence that this peptide cleavage can result in decreased receptor binding activities of human lutropin [126,127] and choriogonadotropin [129].

All β -subunits contain carbohydrate linked to Asn at position 13 and/or 30 (Fig. 2), and the mRNA coding for these subunits in mammals also encodes a 19- or 20-amino-acid pre-sequence. There is greater similarity among β -subunit sequences of mam-

malian follitropin and thyrotropin than among the hormones with lutropin activity (Fig. 2). The β -subunits from four mammals (human, bovine, porcine and rat) contain 66.1 % identical amino acids in their lutropins, whereas their follitropins and thyrotropins respectively exhibit 82.9 % and 82.2 % identity.

The sequence similarity with invariant positions of half-cystine residues in all β -subunits, coupled with their strong binding to the common α -subunit, suggests that disulphide bonds occur in analogous positions in all β -subunits. Experimental evidence for similar disulphide structures of the β -subunits of bovine lutropin and thyrotropin was obtained by a series of partial reductions with dithioerythritol. This resulted first in the opening of a single disulphide bond in each subunit linking residues 93 and 100 [130], and subsequently a second bond in bovine lutropin- β linking residues 26 and 110, and a third bond linking residue 72 with either residue 23 or 88, a specific disulphide interchange having occurred between the last two positions. Further reduction resulted in extensive interchange which prevented additional assignment of disulphides [131]. Cleavage of β -subunits by various chemical and enzymic methods has also led to assignments of disulphide bonds linking residue 93 with 100, 26 with 110 and 23 with 72 in ovine lutropin- β [132,133] and human choriogonadotropin- β [134], but attempts to assign the remaining disulphide bonds by these methods have led to conflicting results between laboratories. A possible additional linkage has been identified in studies of a biosynthetic precursor of human choriogonadotropin β -subunit produced by cultures of the JAR trophoblastic cell line. In this case residues 9, 23, 72, 90, 93 and 100 were not disulphide-linked, which suggested a linkage between residues 9 and 90 in the mature protein in addition to those already assigned [135]. Because of the possibility of transitory formation of 'incorrect' disulphide bridges during folding, as has been observed in pancreatic trypsin inhibitor [136,137], additional evidence will be required to confirm that this latter bond is present in the mature protein.

The similarity between β -subunit sequences from different glycoprotein hormones indicates that all were probably derived from a single ancestral gene via a series of gene duplication events. In addition, the close relationship between human lutropin and human choriogonadotropin β -subunit sequences suggests that genes for the latter have resulted from relatively recent gene duplication events compared with those leading to the genes coding for the β -subunits of thyrotropin, follitropin and lutropin. This is supported by studies locating the human chromosome containing each gene. Those encoding human lutropin and human choriogonadotropin β -subunits were mapped to chromosome 19 [138] where they occurred in a cluster [139], whereas the other subunit genes were each found on different chromosomes. The human follitropin- β gene was on chromosome 11 [92,140], the human thyrotropin- β gene on chromosome 1 [141,142], and the human α -subunit gene on chromosome 6 [138,141]. The presence of the C-terminal extension in the β -subunits of both equine choriogonadotropin and equine lutropin, and its absence from non-equine lutropins, suggests that this extension resulted from a relatively recent mutation which is probably unrelated to the evolution of the human lutropin-human choriogonadotropin gene cluster. Equine choriogonadotropin and lutropin β -subunits possess identical amino acid sequences [78,83] and are encoded by a single gene [143], but there are significant differences in their carbohydrate compositions [144]. The gene for human choriogonadotropin has probably resulted from a single base deletion from an ancestral primate lutropin- β gene causing loss of the termination codon so that the former untranslated region of the gene now codes for protein, as the nucleotide sequences in this region are very similar in the genes coding for human lutropin-

Fig. 2. Comparison of amino acid sequences of β -subunits of human glycoprotein hormones

The sequences have been aligned so that Cys residues coincide, and the numbering above them corresponds to that of choriogonadotropin β -subunit. In order to accommodate a two-residue insertion in thyrotropin- β , gaps indicated by hyphens have been introduced into other β -subunits. Residues invariant in mammalian hormones of similar biological activities are in red, and underlining indicates those which are also invariant in all mammalian β -subunit sequences. References to sources of sequence data are given in the text. Asterisks indicate sites of carbohydrate attachment in human choriogonadotropin- β [33,79,81,115]. In mammals carbohydrate is attached only at Asn-30 in thyrotropin- β [31,104–106,116], at Asn-13 and -30 in follitropin- β [42,94,117–119], and at either residue 13 or 30 in lutropin- β and equine choriogonadotropin- β [47,69,71,77,78,83,120–123]. As discussed in the text, there is good evidence for disulphide bonds linking residue 93 with 100, 26 with 110 and 23 with 72 [130–134] as well as some evidence for a bond linking residue 9 with 90 [135].

β and choriogonadotropin- β [68]. Six genes or pseudogenes coding for human choriogonadotropin- β have been reported [139] and at least five such genes for the corresponding baboon hormone [82], but evidence thus far available indicates that a single gene codes for each mammalian pituitary glycoprotein hormone subunit.

GENERAL STRUCTURAL TYPES OF ATTACHED CARBOHYDRATE

Biosynthesis and types of oligosaccharides in glycoproteins

Studies of the biosynthesis of Asn-linked sugar chains have demonstrated that a common core of $\text{Man}_3\text{GlcNAc}_2$ is transferred *en bloc*, as a still larger unit via a dolichol phosphate intermediate, to the amide group of the appropriate Asn residue of the nascent polypeptide chain [145]. Removal of portions of this larger unit and addition of other sugar residues occur during subsequent processing [146], which often results in branched structures. This processing of N-linked carbohydrate, as well as attachment of sugars to certain serine and/or threonine residues, takes place in the Golgi apparatus [29]. The latter O-linked sugars are attached by O-glycosidic bonds from N-acetyl-galactosamine.

The N-linked sugar chains can be assigned to three sub-groups according to structure (Fig. 3). Those that contain only Man and GlcNAc residues are described as high-mannose type, and are characterized by a heptasaccharide core. Variations occur by the attachment of mannosyl residues linked α 1-2 to the three non-reducing mannosyl termini of the core structure. Complex type oligosaccharides contain a core pentasaccharide and structural changes stem from outer chain moieties linked to its α -mannosyl residues. This subgroup frequently bears a Fuc residue linked to the C-6 position of the proximal GlcNAc and the outer chains often contain the trisaccharide NeuAc-Gal-GlcNAc, but other structures also occur.

The high-mannose type oligosaccharides are considered to be biosynthetic intermediates in the formation of the complex type

sugar chains which have two, three, four or even five branches and are designated respectively as di-, tri-, tetra- and penta-antennary forms. In the tri- and tetra-antennary structures one or two branches are additionally attached to the two α -mannosyl residues, usually with the GlcNAc β 1-4 to the Man α 1-3 and the GlcNAc β 1-6 to the Man α 1-6, but this rule is not invariant [147]. Examples of tri- and tetra-antennary glycans are shown in a later section.

The third category consists of hybrid type oligosaccharides which possess structural features characteristic of both of the above types of sugar chains. They have a common pentasaccharide core and a GlcNAc residue and/or Gal-GlcNAc group linked to an α -mannosyl residue as in complex type sugar chains. Another α -mannosyl residue bears one or two α -mannosyl residues like the high-mannose type and in many cases a β -N-acetylglucosamine residue is linked to the C-4 position of the core β -mannosyl residue. This bisecting GlcNAc residue is also found in some complex type oligosaccharides.

Methods for determination of glycan structure

Methods for the determination of individual sugar components and their linkages within the oligosaccharides have been developed relatively recently compared with procedures for the elucidation of amino acid sequences in a linear chain. The branching that is often found in the glycan moieties has hindered efforts to study their structures in detail. Sugar chains O-linked to serine or threonine can be released by β -elimination with alkaline borohydride and mild heat to form the corresponding alkali-stable sugar alcohols, provided that the amino acid residue is not located in a terminal position [148]. In the case of N-linked oligosaccharides, a considerable number of chemical and enzymic procedures have been developed for their release from glycopeptides and glycoproteins, the choice being influenced by whether protein or oligosaccharide is the product required.

Exoglycosidases in sequence or in mixtures have been used for the deglycosylation of glycoproteins, and more recently various endoglycosidases have been employed [149]. However, the

Fig. 3. General structures of asparagine-linked sugar chains

The asparagine-linked sugar chains can be classified into three subgroups. The high-mannose type glycans contain only mannose and *N*-acetylglucosamine residues attached to a common heptasaccharide core shown in red. Complex type sugar chains contain a pentasaccharide core (shown in red) and structural variations occur through the number and structure of the outer chain moieties attached to the α -mannosyl core residues. Hybrid type oligosaccharides have structures characteristic of high-mannose and complex type sugar chains.

efficiency of hydrolysis is affected by many factors, and recovery is not always quantitative. Specific cleavage of *N*-acetylglucosamine-asparagine bonds by hydrazine obviates most problems raised by the use of enzymes, provided that the procedure is strictly controlled [150].

The analytical methods described by Kobata and his colleagues [150–152] are rigorous, robust and ideal for structural analysis of oligosaccharides, especially those of limited availability; they include paper chromatography, paper electrophoresis and gel-permeation chromatography, and have been successfully applied to the analysis of *N*-linked oligosaccharides from human chorionic gonadotropin [151] and follitropin [153]. Subsequently, the application of h.p.l.c. and lectin affinity chromatography to oligosaccharide analysis obviated the need for the more time-consuming paper chromatographic and electrophoretic steps, and lectin affinity chromatography is now widely accepted for the resolution of complex mixtures of oligosaccharides. This procedure, especially when combined with radiolabelling, facilitates purification of extremely small amounts of material and permits the identification of minor components that might be overlooked by less sensitive analytical methods. The application of sequential lectin chromatography allows one to adduce evidence of structure and expedite detailed structural analysis by more definitive procedures, such as mass spectrometry or ^1H -n.m.r. spectroscopy.

Merkle & Cummings [154] have published a thorough account of the application of lectin affinity chromatography to the analysis

of oligosaccharides derived from glycopeptides and glycoproteins. Serial lectin chromatography is especially valuable because it is rapid and ensures high recovery of labelled substrate. It has been used to complement the analysis by more conventional means of the asparagine-linked sugar chains of human follitropin [153].

Glycans of hormone subunits

Data obtained thus far indicate that the sites of glycosylation of mammalian glycoprotein hormone subunits (with numbering of residues as in Figs. 1 and 2) are as follows. First, all α -subunits have carbohydrate attached at Asn-56 and -82 (Fig. 1). Secondly, all β -subunits have carbohydrate attached at Asn-13 and/or -30 (Fig. 2) with both sites glycosylated in follitropin- β and human chorionic gonadotropin- β . Only Asn-30 is glycosylated in thyrotropin- β and human lutropin- β , but in other mammalian lutropins only Asn-13 is glycosylated. Thirdly, The C-terminal extensions of human and equine chorionic gonadotropin and equine lutropin also contain at least four glycans linked to serine or threonine residues.

Early studies on the carbohydrates of α -subunits revealed that bovine lutropin- α contained more mannose than bovine thyrotropin- α , although their amino acid sequences were identical [155], and the carbohydrate compositions of the common α -subunits of pig lutropin, follitropin and thyrotropin differed in their mannose-to-galactose ratios [156]. More recent work has

confirmed that hormone-specific differences are normally found in the glycans attached to the common α -subunit.

Investigation of the carbohydrate moieties of human choriongonadotropin identified the Asn-linked sugar components of the α -subunit as Gal, Man, GlcNAc and NeuAc [157] in the complex type structure of Fig. 3 [151,158,159]. The Asn-linked carbohydrate of the β -subunit contains these same sugars, as well as fucose linked to the C-6 position of the proximal GlcNAc [151,158,159]. These variations, as well as incomplete glycosylation of a proportion of the molecules, result in heterogeneity of the hormone and of its individual subunits. The Ser-linked carbohydrate contains NeuAc, Gal, GlcNAc and GalNAc, and these structures are also heterogeneous with certain sugar residues missing in some molecules [81,160].

Cole *et al.* [160] described di-, tri-, tetra- and hexa-saccharide structures in a tryptic peptide that contained three of the O-linked glycans of human choriongonadotropin β -subunit. In equine choriongonadotropin the major O-linked glycan was the trisaccharide NeuAc α 2-3Gal β 1-3GalNAc, although minor amounts of tetra-, penta- and hexa-saccharides were present, as well as oligo-*N*-acetyl-lactosamine structures [161], which had previously been proposed by Bahl & Anumula [162].

It has been known for some time that the carbohydrates of human choriongonadotropin isolated from women with hydatidiform mole or with choriocarcinoma differ from those of the hormone obtained from healthy subjects [163–165], and in a comparative study of three hormonal preparations from those sources Amano *et al.* [166] noted that hormonal activity decreased with altered glycosylation.

In 'free α -subunit' isolated from pregnancy urine there was more fucose and *N*-acetylglucosamine as well as 2.5-fold more sialic acid and galactose than in the subunit obtained from intact human choriongonadotropin [66]. In addition, 'free α -subunit' encountered in certain pathological conditions differs from that found in pregnancy. The sugar moieties of this subunit isolated from the urine of a healthy pregnant woman contained only diantennary complex type N-glycans, whereas those derived from a patient with adenocarcinoma also contained tri- and tetra-antennary sugar chains [167].

Hormone and species specificity of N-glycosylation

The limited availability of purified pituitary glycoprotein hormones and the more complex and heterogeneous nature of their oligosaccharides precluded their rigorous characterization until recently. Earlier studies of follitropin and lutropin from human, ovine and bovine pituitary glands [168–170], which employed conventional methods of structural analysis, did not provide definitive structures but did reveal some important features, particularly the terminal sulphation of glycans found in ovine, bovine and human lutropin.

For some years structural investigations of the N-glycans of the anterior pituitary glycoprotein hormones were hindered by the inadequacy of procedures for the separation of sulphated and sialylated oligosaccharides. This limited the application of ^1H -n.m.r. spectroscopy, at present the most rigorous method for the structural analysis of the more common sialylated glycans. Furthermore, in the absence of suitable sulphatases, there was no effective means for the removal of sulphate residues without damage to the underlying carbohydrate structures.

These problems have been largely resolved with the development of h.p.l.c. procedures and methanolysis for the removal of sulphate. Anion-exchange h.p.l.c. was introduced for the separation of anionic oligosaccharides according to the number and types of acidic moieties [171], and ion-suppression amine adsorption h.p.l.c. was applied to the resolution and

characterization of different types of negatively charged sugar chains [172].

The application of such procedures, coupled with lectin affinity chromatography and endo- and exo-glycosidase digestion enabled Green *et al.* [173] to examine structures of metabolically labelled oligosaccharides from bovine lutropin. The N-glycans of that hormone were a mixture of neutral, mono- and di-sulphated species. Carbohydrate structures similar to those linked to Asn in human choriongonadotropin were observed, except that NeuAc-Gal- was replaced by sulphate-GalNAc- [174,175]. It has previously been shown for the human hormones that sialic acid is present in relatively high concentrations in choriongonadotropin [176] and follitropin [177], and that lesser amounts are present in lutropin [178].

Green *et al.* [179] and Green & Baenziger [175] reported that ovine and human follitropin and lutropin, as well as bovine follitropin, contain sulphate at the termini of some carbohydrate chains, in structures similar to those found in bovine lutropin. Other oligosaccharides, similar to those found in N-glycans of human choriongonadotropin, contain chain-terminating sialic acid. However, there are differences between hormones and between mammalian species in the relative amounts of sulphated and sialylated chains in the pituitary hormones. For each of the three species studied, the ratio of sulphated to sialylated carbohydrate chains was more than 10-fold higher in lutropin than in follitropin, and human follitropin contained mainly sialylated structures with very little of the sulphated forms [173,180]. Within each species, differences were found between the carbohydrate moieties of the α -subunits in each hormone, even though the amino acid sequences were the same.

Detailed structural analysis of the N-glycans of intact human follitropin [153] demonstrated that 95% of its four N-glycans were acidic oligosaccharides that contained between one and three sialic acid residues (Fig. 4). The asialo sugar chains were a heterogeneous mixture of di-, tri- and tetra-antennary complex-type oligosaccharides with and without a fucose residue linked α 1-6 to the proximal GlcNAc. Bisecting GlcNAc was found in 30% and 15% of the di- and tri-antennary structures, respectively [153].

The monoantennary sugar chain found in human choriongonadotropin was not detected in follitropin from human glands. Di- and tri-antennary structures predominated, and core fucosylation was noted in about 50% of the total oligosaccharides. The structures of the N-glycans of human follitropin proposed by Green & Baenziger [175,180] were similar to those described by Renwick *et al.* [153], in that the major constituents were di- and tri-antennary complex chains. The precise structures and distribution were significantly different, particularly in the case of the triantennary oligosaccharides, and the former workers did not report the presence of tetra-antennary chains. Both α 2-3 and α 2-6 linked sialic acids were identified with the former predominant in human follitropin [180], but their precise positions and linkages in each oligosaccharide have not yet been determined. Examination of the acidic sugar chains from bovine and ovine follitropin (about 70% of the total in each case) revealed that the latter contained mostly diantennary complex-type oligosaccharides while the former had both di- and tri-antennary chains [180]. In contrast to the human hormone, bovine and ovine follitropin contained significant amounts of sulphated oligosaccharides (60% and 20% of the acidic glycans respectively). Thus the N-glycans show considerable variation between species in their branching structures and in respect of terminal sialylation and sulphation.

The N-glycans of bovine and human thyrotropin have been investigated by Green & Baenziger [175,180] and the bovine hormone, like lutropin from the same source, contained sulphated

Structure	Relative abundance (%)	
	With proximal fucose	Without fucose
<div><div>Galβ1-4GlcNAcβ1-2Manα1</div><div>6</div><div>Manβ1-4GlcNAcβ1-4GlcNAc</div><div>3</div><div>±Fucα1</div><div>6</div></div>	21	11
<div><div>Galβ1-4GlcNAcβ1-2Manα1</div><div>6</div><div>Manβ1-4GlcNAcβ1-4GlcNAc</div><div>3</div><div>GlcNAcβ1</div><div>4</div><div>±Fucα1</div><div>6</div></div>	7	7
<div><div>Galβ1-4GlcNAcβ1-2Manα1</div><div>6</div><div>Manβ1-4GlcNAcβ1-4GlcNAc</div><div>3</div><div>GlcNAcβ1</div><div>4</div><div>Galβ1-4GlcNAcβ1</div><div>4</div><div>Manα1</div><div>3</div><div>±Fucα1</div><div>6</div></div>	4	3
<div><div>Galβ1-4GlcNAcβ1</div><div>6</div><div>Manα1</div><div>2</div><div>Manβ1-4GlcNAcβ1-4GlcNAc</div><div>3</div><div>±Fucα1</div><div>6</div></div>	8	9.2
<div><div>Galβ1-4GlcNAcβ1-2Manα1</div><div>6</div><div>Manβ1-4GlcNAcβ1-4GlcNAc</div><div>3</div><div>Galβ1-4GlcNAcβ1</div><div>4</div><div>Manα1</div><div>2</div><div>±Fucα1</div><div>6</div></div>	8	15.3
<div><div>Galβ1-4GlcNAcβ1</div><div>6</div><div>Manα1</div><div>2</div><div>Manβ1-4GlcNAcβ1-4GlcNAc</div><div>3</div><div>Galβ1-4GlcNAcβ1</div><div>4</div><div>Manα1</div><div>2</div><div>Galβ1-4GlcNAcβ1</div><div>4</div><div>Manα1</div><div>2</div><div>±Fucα1</div><div>6</div></div>	5	—

Fig. 4. N-linked oligosaccharides of human follitropin

The Asn-linked oligosaccharides of intact follitropin were obtained as their tritiated and deuterated alditols after hydrazinolysis; 95 % were acidic and were totally converted to a mixture of neutral oligosaccharides on treatment with sialidase. The mixture was then resolved into six fractions by lectin affinity chromatography performed in series and the fractions were analysed by sequential exoglycosidase digestion, in conjunction with column chromatography on Bio-Gel P-4. Confirmatory data were obtained by methylation analysis and gas-liquid chromatography/mass spectrometry. Full details were published by Renwick *et al.* [153].

oligosaccharides almost exclusively, which were mostly derived from the diantennary disulphated structure. The varieties of sialylated and sulphated N-glycans in human thyrotropin were similar to those of human lutropin except for their higher content of sulphate and the bisected structures found in the sialylated glycans of the latter hormone were not present

Although there are significant differences in type and distribution of sialylated and sulphated structures among the

glycoprotein hormones of human and animal origin, there are also structural similarities. The acidic oligosaccharides of human lutropin and its placental analogue, choriogonadotropin, have di- and mono-antennary complex structures in a ratio of 4:1, although some of them are bisected in the case of lutropin. The chief differences in branch structures occur in terminal sialylation/sulphation and in their sialic acid linkages. Follitropin, lutropin and thyrotropin of human origin contain a

Structure	Relative abundance (%) in:		
	hLH α Asn-56	Asn-82	hLH β Asn-30
SO ₄ -4GlcNAc β 1-4GlcNAc β 1-2	7	2	10
NeuAc α 2-3Gal β 1-4GlcNAc β 1-2	18	5	15
NeuAc α 2-6Gal β 1-4GlcNAc β 1-2	5	2	4
Gal β 1-4GlcNAc β 1-2	5	4	10
GlcNAc β 1-2	4	<2	3
H	9	3	5
Man α 1-3	5	<2	<2
SO ₄ -4GalNAc β 1-4GlcNAc β 1-2			
NeuAc α 2-3Gal β 1-4GlcNAc β 1-2	5	6	7
NeuAc α 2-6Gal β 1-4GlcNAc β 1-2	2	4	<2
Gal β 1-4GlcNAc β 1-2	3	4	4
H	5	3	4
NeuAc α 2-6Gal β 1-4GlcNAc β 1-2			
NeuAc α 2-3Gal β 1-4GlcNAc β 1-2	2	12	3
Gal β 1-4GlcNAc β 1-2	3	6	2
H	2	4	<2
NeuAc α 2-6GalNAc β 1-4GlcNAc β 1-2			
Tricharged oligosaccharides	2	4	6
Neutral oligosaccharides	9	12	7

Fig. 5. N-linked oligosaccharides at individual glycosylation sites in human lutropin

The N-linked oligosaccharides were liberated by hydrazinolysis from the intact β -subunit of human lutropin and from glycopeptides obtained after tryptic digestion of the α -subunit, followed by reduction and fractionation as alditols by anion-exchange and ion-suppression amine adsorption h.p.l.c. They were mainly identified by one- and two-dimensional ^1H -n.m.r. spectroscopy at 400 MHz, supplemented by composition analysis, methylation analysis and mass spectrometry. Relative abundances are expressed as percentages of total oligosaccharides at each glycosylation site. The individual structures are composed of one of the three core structures (shown in red) extended at the α 1-6 linked mannose residue by the listed sequences. Full details were published by Weisshaar *et al.* [188]. The numbering of the glycosylated asparagine residues is the same as that in Figs. 1 and 2.

high proportion of diantennary oligosaccharides of the complex type but these differ in patterns of terminal sialylation/sulphation. The heterogeneity in the carbohydrate of each glycoprotein hormone results in variability in the content of negatively-charged sulphate and/or sialic acid groups and causes charge heterogeneity. The missing residues at the termini of some of the polypeptide chains as well as pre-existing internal cleavages contribute to a lesser extent to the charge heterogeneity of these hormones.

DETAILED SITE-SPECIFIC STUDIES OF CARBOHYDRATE STRUCTURES

Despite the availability of a considerable amount of information concerning the structures of the carbohydrate components of the glycoprotein hormones, there was a need for more rigorous analysis and the assignment of specific structures to particular amino acid residues in each subunit. This requirement became more urgent with the growing perception that the actions of enzymes concerned in oligosaccharide processing are influenced by the protein structures of the α - β heterodimer and that glycans attached to specific Asn residues appear to have particular roles in signal transduction. The N-glycans of human choriogonadotropin were the first oligosaccharides of a glycoprotein hormone to be characterized in detail. More than 90 % of the asparagine-linked sugar chains of this hormone were found to be sialylated, mono- and diantennary complex type structures [158]. There was some core-fucosylation of the diantennary chains and all the sialyl residues were α 2-3 linked to galactose, in contrast to many serum

glycoproteins where the α 2-6 linkage predominates. Identical disialylated structures were proposed for the four N-glycans by Kessler *et al.* [159] who found fucose only in glycopeptides derived from the β -subunit of human choriogonadotropin. Mizuochi & Kobata [151] subsequently examined the distribution of specific oligosaccharides on the α - and β -subunits of human placental choriogonadotropin, and found that the α -subunit contained mono- and diantennary structures in equal amounts, whereas the β -subunit possessed two diantennary glycans in equal quantities. Based on these results, site-specific distribution of the three structures on human placental choriogonadotropin was proposed, with monoantennary sugar chains only in the α -subunit and fucosylated chains only in the β -subunit. When the hormones purified from placenta and urine were compared, they were almost identical except that the former contained more neutral oligosaccharides than the latter. The results of analysis of the O- and N-glycans of human urinary choriogonadotropin by ^1H -n.m.r. spectroscopy at 500 MHz [181] were in general agreement with those of Mizuochi & Kobata [151], although there were differences in the precise structures attached to the β -subunit. Methods for the purification of glycoprotein hormones, which employed ion-exchange chromatography, had inherent disadvantages because of the considerable charge heterogeneity of these hormones. The overlapping charges of the three pituitary glycoprotein hormones create difficulties in their complete separation, and the purification of these hormones as single fractions is not always possible by such methods [182,183]. In addition, certain charged forms of these hormones may be selected during ion-exchange chromatography [184], and this

Structure	Relative abundance (%) in:			
	hCG α		hCG β	
	Asn-56	Asn-82	Asn-13	Asn-30
<div>NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1 6 Manβ1-4GlcNAcβ1-4GlcNAcβ1-NAsn 3 [Fucα1 6]</div>	18	61	79	91
<div>NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1 Manα1 6 Manβ1-4GlcNAcβ1-4GlcNAcβ1-NAsn 3</div>	50	39	≤ 7	≤ 3
<div>NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1 Manα1 3 6 Manβ1-4GlcNAcβ1-4GlcNAcβ1-NAsn 3</div>	19	-	-	-
<div>NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1 Manα1 6 Manα1 3 6 Manβ1-4GlcNAcβ1-4GlcNAcβ1-NAsn 3</div>	10	-	-	-

Fig. 6. N-linked oligosaccharides at individual glycosylation sites in human chorionic gonadotropin

Glycopeptides that contained individual N-glycosylation sites of human chorionic gonadotropin were obtained from the α -subunit (N-glycosylated at Asn-56 and Asn-82) and from the β -subunit (N-glycosylated at Asn-13 and Asn-30) by digestion with trypsin and chymotrypsin, respectively. The glycopeptides were analysed by one- and two-dimensional ^1H -n.m.r. spectroscopy at 400 MHz after purification by reverse-phase h.p.l.c. and identification by amino acid sequencing. The relative abundances of the carbohydrate components of the α -subunit were determined by integration of ^1H -n.m.r. spectra, whereas those on the β -subunit were measured by ^3H -counting of oligosaccharide alditols resolved by analytical anion-exchange h.p.l.c. Full details were published by Weisshaar *et al.* [190]. The numbering of the glycosylated asparagine residues is the same as that of Figs. 1 and 2.

could further complicate the characterization of heterogeneous forms and the structural analysis of their carbohydrate components.

Procedures were therefore developed for the purification of human lutropin and thyrotropin by hydrophobic chromatography, in which the effect of charge on their separation is minimal [185]. Purified hormones prepared as single fractions in good yield and with high biological activity were obtained and used as starting materials for the elucidation of their carbohydrate structures. Reversed-phase h.p.l.c. at pH 6.5 was employed for complete separation of the subunits of each of the hormones after dissociation with guanidine HCl [186].

Weisshaar *et al.* [187,188] isolated the N-linked oligosaccharides from tryptic digests of the α -subunits of ovine and human lutropin, and from each β -subunit. These were individually subjected to hydrazinolysis, and the oligosaccharide products were reduced to their alditols with sodium borohydride [187,188]. Each was then fractionated by anion-exchange and ion-suppression amine-adsorption h.p.l.c. [189]. In the case of human choriogonadotropin, ^1H -n.m.r. spectroscopy was performed on intact glycopeptides isolated after tryptic digestion of each subunit, which obviated the requirement for extensive chromatographic resolution before analysis.

The N-glycans of ovine lutropin were examined by ^1H -n.m.r. spectroscopy at 400 MHz [187]. Monosulphated, mostly hybrid type, oligosaccharides predominated at both glycosylation sites in the α -subunit, whereas a disulphated diantennary complex type structure accounted for more than 60% of the total oligosaccharides in the β -subunit. Moreover, the N-glycans

attached to the β -subunit were almost completely fucosylated (Fuc α 1-6) at the reducing terminal GlcNAc, whereas those in the α -subunit were either approximately 50% fucosylated (Asn-82) or were fucosylated only to a minor degree (Asn-56). The results clearly indicated distinct subunit- and site-specific synthesis of the oligosaccharides on ovine lutropin and suggested that biosynthesis is effectively influenced by the surrounding polypeptide chain(s) at a given site.

Detailed investigation of the N-linked oligosaccharides of human lutropin [188] revealed predominantly diantennary complex type structures at all three glycosylation sites (Fig. 5). It should be noted that the numbering of α -subunit glycosylation sites used in this Review is that of bovine- α with gaps introduced in the human- α sequence at residues 5–9 (Fig. 1). (The glycosylation sites of the latter are actually at its Asn residues 52 and 78). Glycans attached to Asn-56 in the α -subunit and Asn-30 in the β -subunit showed a remarkably similar pattern, with mainly chain-terminating 4-sulphated GalNAc and a sulphated/sialylated structure as the major single component. Virtually all N-glycans on the β -subunit bore a fucose residue linked α 1-6 to the proximal GlcNAc, whereas those at Asn-56 and Asn-82 of the α -subunit were mainly non-fucosylated. The oligosaccharides at Asn-82 in the α -subunit were sialylated rather than sulphated and contained the unique sequence NeuAc α 2-6GalNAc β 1-4GlcNAc β 1-2Man α 1-3 as a component of most mono- and disialylated compounds.

Wiesshaar *et al.* [190] studied glycopeptides that contained the four N-glycans of human chorionic gonadotropin obtained from proteolytic digests of each subunit. Each glycopeptide was

Fig. 7. N-linked oligosaccharides at individual glycosylation sites in human thyrotropin

Human thyrotropin was dissociated into its subunits and the α component (glycosylated at Asn-56 and Asn-82) was treated with trypsin to yield two glycopeptides which were subsequently separated by reverse-phase h.p.l.c. and identified by amino acid sequence analysis. The oligosaccharides were released from the glycopeptides and from the intact β -subunit by hydrazinolysis and were fractionated as alditols by anion-exchange and ion-suppression amine-adsorption h.p.l.c. preparatory to analysis by one- and two-dimensional ^1H -n.m.r. spectroscopy at 400 MHz. Relative abundances are expressed as percentages of total oligosaccharides at each site. The core structure, which is common to all identified components, is extended by the listed sequences; minor (e.g. monosialylated) oligosaccharides make up the total of 100%. Full details were published by Hiyama *et al.* [192]. The numbering of the asparagine residues is the same as that of Figs. 1 and 2.

subjected to one- and two-dimensional ^1H -n.m.r. spectroscopy, and it was found that oligosaccharides attached to Asn-56 of the α -subunit comprised monosialylated monoantennary, disialylated diantennary and two types of monosialylated hybrid type structures in a ratio approaching 5:2:3 (Fig. 6). Asn-82 of the α -subunit bore monoantennary and diantennary structures almost exclusively, in a ratio of about 2:3, and both N-glycosylation sites in the β -subunit contained the disialylated diantennary structure, partially (about 25%) and completely α 1-6 fucosylated at the GlcNAc residue linked to Asn-13 and Asn-30 respectively. Thus the monoantennary complex type structure was present only in the α -subunit at both glycosylation sites.

The distinct site-specific distribution of the oligosaccharide structures among individual glycosylation sites of human chorionic gonadotropin appeared primarily to reflect the influence of the surrounding protein structure on the substrate accessibility of the Golgi processing enzymes α -mannosidase II, GlcNAc transferase II and α 1,6-fucosyltransferase.

The N-glycan(s) at Asn-56 in the α -subunit of human choriogonadotropin have been implicated in the biological action of the intact hormone [191], but more definitive studies are required before biological activity can be assigned to particular structures in this and other glycoprotein hormones.

Hiyama *et al.* [192] examined the N-glycans attached at each of the three glycosylation sites of human thyrotropin by ^1H -n.m.r. spectroscopy and reported that they were mainly diantennary complex type structures with a common Man α 1-3 branch that terminated in 4-O-sulphated GalNAc (Fig. 7). The relative amounts of the two major complete diantennary oligosaccharides and their core-fucosylation differed according to glycosylation site; the sulphated/sialylated diantennary oligosaccharide was most abundant at the two sites on the α -subunit, whereas the disulphated, core-fucosylated oligosaccharide was more plentiful on the β -subunit. Some interesting structural features previously not reported for the N-glycans of human thyrotropin included 3-O-sulphated galactose (SO₄-3Gal) and peripheral fucose (Fuc α 1-3GlcNAc) in the Man α 1-6 branch of some diantennary structures; the former suggests the presence of a hitherto uncharacterized galactose-3-O-sulphotransferase in thyrotroph cells of the human pituitary gland.

FUNCTIONS OF THE CARBOHYDRATE COMPONENTS

A detailed discussion of structure–function relationships of the protein portions of these hormones is beyond the scope of this article. For additional information the interested reader is referred to recent publications on this subject [2,3,28].

The significance of sialic acid residues in the metabolism of glycoprotein hormones has been recognized for more than 30 years [193–195], and terminal sialylation is thought to protect the hormones from rapid clearance from the circulation by lectins that bind asialoglycoproteins [196,197]. For example, the half-life of human choriogonadotropin in the rat was reduced from 50 min to 1 min after desialylation with neuraminidase [196]. In bovine and ovine lutropin, which contain sulphate groups and little or no sialic acid, the former residues are thought to play a comparable role in metabolic clearance.

Several sets of experiments with human choriogonadotropin have suggested that sialic acid residues are necessary for full expression of hormonal activity because desialylation reduces this property *in vitro* [198–200]. On the other hand, recombinant bovine lutropin that contained sialylated oligosaccharides in contrast to the native hormone was reported to have increased steroidogenic activity after desialylation [201]. Furthermore, Amir *et al.* [199] reported that desialylation of human choriogonadotropin enhanced its avidity for its receptors and changed this hormone from a full to a partial agonist in testis. It was suggested that sialic acid in its β -subunit has a dominant role in evoking these effects.

Studies of subunit synthesis suggest that their attached carbohydrate facilitates proper disulphide bond formation during folding of the nascent peptide chains and prevents proteolysis and aggregation of subunits. Thus inhibition of glycosylation with tunicamycin resulted in mouse thyrotropin subunits which aggregated intracellularly and were readily degraded by proteolytic enzymes [202], and synthesis of bovine α -subunit in a cell-free system or in *Escherichia coli* led mainly to incorrect folding of non-glycosylated polypeptide chains [203], although small amounts of native-like structure were produced [204]. Similarly, site-directed mutagenesis to prevent carbohydrate attachment at either or both of the Asn-linked glycosylation sites in human choriogonadotropin- α , by substituting Asp for Asn-56 or -82 (Fig. 1) or Ala for Thr-58 or -84 (Fig. 1), indicated that only the carbohydrate at position 56 was necessary to facilitate proper folding so that association with β -subunit could occur. The carbohydrate at position 82 was important in preventing degradation of the subunit [191]. The choice of residues to be substituted was based on the requirement of the sequence Asn-

Xaa-Thr or Asn-Xaa-Ser (where Xaa is any amino acid) for enzymic N-glycosylation of proteins [205–207].

Reassociated heterodimers of chemically deglycosylated ovine lutropin- α with native or deglycosylated ovine lutropin- β or native ovine follitropin- β possessed enhanced receptor binding activity compared with the native hormones, but they were virtually inactive in stimulating cyclic AMP production in target cells, and they inhibited this activity of the native hormone. On the other hand, the heterodimer of ovine lutropin- α and deglycosylated ovine lutropin- β had nearly full activity in adenylate cyclase stimulation, suggesting that the carbohydrate of the α -subunit plays a dominant role in the transduction of the hormone signal into the cell [208]. This is supported by other studies employing site-directed mutagenesis and gene transfer to obtain mutants of human choriogonadotropin lacking individual glycosylation sites. Mutant forms containing no carbohydrate had significantly reduced activity in stimulation of cyclic AMP or steroidogenesis, and this was due mainly to the lack of oligosaccharide at the α -subunit Asn-56 (Fig. 1) [209,210]. Mutant forms lacking all carbohydrate or with oligosaccharide attached only at the α -subunit Asn-82 (Fig. 1) also acted as competitive antagonists. Similar approaches have revealed that O-glycans attached to the C-terminal extension of the β -subunit of human choriogonadotropin are not important for receptor binding or signal transduction, but appear crucial for *in vivo* biological activity [211].

Native human choriogonadotropin that was bound to rat testis receptors could still bind monoclonal antibodies specific for two regions of the hormone. However, deglycosylated hormone bound to the receptors with a 4-fold increase in affinity and was inaccessible to the same antibodies, suggesting that the presence of carbohydrate prevents formation of additional hormone–receptor contacts which result in loss of cyclic AMP stimulating activity [212].

Hattori & Wakabayashi [213] used inhibitors of oligosaccharide processing to assess the contributions of high-mannose, hybrid and complex type N-glycans to the biological activity of rat lutropin. Hormonal preparations that contained high-mannose and hybrid type oligosaccharide chains were 40–60% less potent than lutropin with N-glycans of the complex type in their stimulation of adenylate cyclase in Leydig cells, whereas their receptor-binding capabilities were significantly increased.

Amano *et al.* [166] compared the production of cyclic AMP by preparations of choriogonadotropin derived from healthy women and those with hydatidiform mole and choriocarcinoma, which contain altered N-glycans [163,214]. Complete desialylation resulted in a marked reduction of this biological activity in all three preparations, and the activity of choriogonadotropin in the pathological conditions was 20–30% lower than that shown by hormone obtained from healthy women. However, the influence of different neutral oligosaccharide structures was small in comparison with that exerted by terminal sialic acids.

Although these studies suggest that incomplete or modified processing of N-glycans impairs the full expression of the hormonal activity of rat lutropin and human choriogonadotropin, other findings indicate that the effects of oligosaccharide structure may differ between hormones. Unlike human choriogonadotropin, desialylated rat lutropin showed increased receptor-binding and enhanced biological activity [215].

The available evidence thus points to a major functional role for the carbohydrate component of glycoprotein hormones, particularly that of the α -subunit, in the coupling of the hormone–receptor complex to the hormonal response. This, together with the strong sequence homology of α -subunits throughout vertebrate evolution, indicates a basic function for

this component when combined with any of the β -subunits. In addition to their roles in regulation of metabolic clearance, facilitation of proper folding of subunits during synthesis, control of hormonal response and modulation of hormone potency, it has been suggested that the oligosaccharides may also be important in intracellular sorting of hormones into separate secretory granules [216].

Calvo & Ryan [217] proposed that a membrane lectin may be involved in the regulation of luteal cell choriogonadotropin-stimulated adenylate cyclase, and they speculated that cross-linking of receptor and lectin may be involved in micro-aggregation, stimulation of adenylate cyclase and internalization. The hybrid and monoantennary N-glycans attached to the α -subunit of human choriogonadotropin contain α -mannosyl residues and thus could conceivably interact with a mannose-specific lectin. Membrane lectins that are highly specific for residues such as mannose and galactose have been identified in mammalian cells and tissues [218], and Drickamer [219] has discussed two distinct classes of carbohydrate-recognition domains in animal lectins. Although no membrane lectin has yet been described, the choriogonadotropin/lutropin receptor contains a 10-residue amino acid sequence found in soy bean lectin [220]. More recently, Thotakura and his colleagues [221] obtained glycopeptides and oligosaccharides from human chorionic gonadotropin and other glycoproteins and showed that those preparations inhibited gonadotropin-stimulated adenylate cyclase, as well as the binding of that hormone to its receptor, in contrast to the observations of Calvo and Ryan [217]. On the basis of their experiments, Thotakura *et al.* [221] proposed that N-linked oligosaccharides from various glycoproteins perturb the interactions of that hormone with rat ovarian receptors through a supposed carbohydrate-binding site on the receptor. Information on the structure of this receptor has been reviewed by Segaloff *et al.* [222].

Renwick & Wiggins [223] have considered the role of N-glycans in the biological actions of human choriogonadotropin and lutropin, which interact with a common receptor with equal avidity. Although there are major differences between the structures of the peripheral carbohydrate chains of these hormones [188,190], the presence of chain-terminating negatively charged groups in both has led to a proposal that all glycoprotein hormones may function by interaction with structured water in the receptor cavity [223].

FINAL COMMENTS AND FUTURE DEVELOPMENTS

Structural analysis of intact glycoprotein hormones has so far defied elucidation; the size and complexity of each hormonal subunit preclude analysis by high-field n.m.r. X-ray diffraction data are limited at present but the crystallization of deglycosylated and desialylated forms of human chorionic gonadotropin [224,225] is a major achievement. It should eventually lead to an accurate three-dimensional structure of this protein, which will be generally applicable to other glycoprotein hormones and which will result in unambiguous identification of their disulphide bonds.

The application of new, rigorous, analytical techniques for the structural determination of complex carbohydrates [147,226,227] has greatly facilitated the acquisition of detailed information concerning the N-linked oligosaccharides at individual glycosylation sites in ovine and human lutropin [187,188], human chorionic gonadotropin [190] and human thyrotropin [192]. Similarly, many investigations have probed salient features of the protein backbones of these hormones using circular dichroism [228,229], monoclonal antibodies [230–235] and synthetic

peptides [236–239], especially domains that appear to be vital in hormonal receptor interactions.

The available evidence supports the view that the glycoprotein hormones consist of two dissimilar subunits associated by hydrophobic interaction with their N-linked oligosaccharides closely apposed to the protein surface, such that they form an incomplete calyx that permits exposure of hydrophobic domains on both subunits. This perception is based on the observations that the α 1-6 bond of di- or tri-antennary oligosaccharides is flexible [240–246] and that analytical centrifugation of native and deglycosylated hormones revealed essentially no difference in sedimentation constant [247].

While refinement of force-field programmes for the calculation of energetically favoured conformations of glycopeptides [245] should lead to computer-generated structures of hormonal subunits in the foreseeable future, experimentally derived data related to the three-dimensional structures of the glycosylated and deglycosylated hormonal heterodimers are obligatory. Even then, heterogeneity of the attached oligosaccharides, particularly those that are N-linked, will remain problematic.

We cannot yet state which specific carbohydrate components help to confer biological activity. Anterior pituitary hormones extracted from animal glands are almost exclusively obtained from both sexes, of variable age and reproductive status (especially in humans), collected at indeterminate times after death and presumably containing hormone at different stages of biosynthesis. Such factors are impossible to control in the human and probably account for a greater degree of structural heterogeneity than the microheterogeneity inherent in oligosaccharide biosynthesis.

The N-linked oligosaccharides of ovine lutropin and human chorionic gonadotropin were less heterogeneous than those derived from human pituitary glycoprotein hormones [187,190] and the chorionic gonadotropin would be more valuable if it could be obtained from a single source. Hormone obtained from pooled urine could well contain carbohydrate derived from malignant tissue [248] and while glycosylation patterns may not be identical with those of human origin, recombinant species if available in sufficient quantity may yield more consistent information [249] and provide a structurally well-defined molecule whose carbohydrate and protein domains could be more accurately related to biological activities such as receptor-binding [250] and changes associated with the hormonal response [251,252].

It is a pleasure to acknowledge the helpful and stimulating discussions between A.S.H. and Drs. Murray and Francesca Stewart in the early stages of writing this Review, as well as the valuable criticism provided to A.G.C.R. by Dr. J. Hiyama.

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