

Phosphorylation of human plasma α_2 -Heremans–Schmid glycoprotein (human fetuin) *in vivo*

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A fraction of α_2 -Heremans–Schmid (α_2 -HS) glycoprotein (human fetuin) isolated from plasma was phosphorylated at serine-120 and serine-312 as shown by MS and peptide fragment sequencing after tryptic digestion. Serine-312-containing peptides were phosphorylated to 77% as determined from relative peak heights in the mass spectrum, which together with the phosphorylation of serine-120 implies a molar degree of phosphorylation of at least 1. Approximately 20% of the circulating fetuin plasma pool was phosphorylated to approx. 1 mol of phosphate/mol of protein. The remainder did not contain phosphate, resulting in an average phosphorylation degree for the protein in plasma of approx. 0.2 mol/mol. The isolated α_2 -HS glycoprotein was a heterodimer in which the entire C-terminal part of the connecting

peptide including threonine-321 was present, but traces of C-terminally trimmed connecting peptide fragments were also found. The short B-chain was O-glycosylated to approx. 40%, whereas the N-glycosylation of asparagine-138 and asparagine-158 seemed to be 100%. This finding, for the first time, that circulating human plasma fetuin is partly phosphorylated, implies that the effects of phosphorylated α_2 -HS glycoprotein on insulin signal transduction seen in different cell systems could be relevant to its physiological function *in vivo*.

Key words: glycosylation, MS, phosphate analysis, phosphopeptide analysis.

INTRODUCTION

Fetuin is a family of proteins collectively named after the bovine counterpart fetuin first described by Pedersen [1]. The human counterpart of fetuin, α_2 -Heremans–Schmid glycoprotein (AHSG) is secreted mainly by the liver and is present in the blood of adults at an average concentration of 0.3–0.6 mg/ml.

Olivier et al. [2] found a second member of the mammalian fetuin family in rat, mouse and human on the basis of domain homology, overall conservation of cysteine residues and chromosomal assignments of the corresponding genes. They named this protein fetuin-B, as opposed to the previously known human variant AHSG called fetuin-A in their paper.

AHSG is a negative acute-phase protein in man, both *in vivo* [3,4] and in experimental models with the human hepatoma cell lines HepG2 or Hep3B [5–7]. The function of this protein still remains elusive but it has been suggested that AHSG is involved in various functions, some similar to those found in other mammals (reviewed in [8]). Apart from its function in bone, a common observation is that the protein must be phosphorylated to be physiologically active in mammals, because the non-phosphorylated version has minimal or no effects [9–12].

Bovine serum fetuin [13,14] and rat serum fetuin (pp63) [15] are phosphorylated but, to our knowledge, AHSG has never been found in a phosphorylated form in human plasma or serum, although attempts have been made to determine phosphate in human AHSG, either directly [10,16] or during sequence analysis of the protein [17]. However, human AHSG is secreted in a partly phosphorylated form from the human cell line HepG2 [18], from Hep3B cells and as overexpressed recombinant human AHSG in insect cells [10,19]. The latter AHSG inhibits insulin-induced autophosphorylation of the insulin receptor [12,19–22].

Among the mammalian fetuins, human plasma AHSG is unique in that it is a two-chain protein, whereas all other known

mammalian fetuins consist of a single chain [23]. However, the cell-culture-derived and recombinant human AHSG are secreted in a one-chain form [18,21] in addition to the two-chain form isolated from human serum [24–26]. The one-chain form contains 349 amino acid residues, whereas the two-chain form consists of one A-chain and one B-chain; the heavy A-chain contains residues 1–282 and the so-called connecting peptide consists of residues 283–321. The light B-chain comprises residues 323–349 of the one-chain form (a graphic presentation of the amino acid sequence can be seen in the Results section). The two-chain form is opened between arginine-322 and threonine-323 and a disulphide bridge between cysteine-14 and cysteine-340 joins the A-chain and the B-chain. The whole connecting peptide has been reported to be present in full apart from arginine-322 [17] or to be C-terminally digested to various degrees, as in patients with sepsis [27].

In a previous study [28] we determined the degree of phosphorylation of human plasma fibrinogen from patients undergoing thrombolytic treatment. In the initial stages of that study we attempted to purify fibrinogen from human plasma by affinity chromatography on commercially available protamine–agarose (Sigma). We found that one (or more) phosphorylated protein was purified together with the fibrinogen. The problem with the purification of fibrinogen was overcome by using protamine–agarose made in our laboratory as described by Dempfle and Heene [29]. The unknown protein was eluted at high salt concentration (700 mM NaCl) from this protamine gel, preceding the elution of fibrinogen.

We have now identified the unknown phosphorylated protein as AHSG (human fetuin). It contained phosphate on serine-120 located in the A-chain and on serine-312 located in the connecting peptide; i.e. at two of the phosphorylation sites suggested by Jahnen-Dechent et al. [18]. The average degree of phosphorylation in plasma was at least 0.2 mol of phosphate/mol of

Abbreviations used: 7-AHA, 7-aminoheptanoic acid, AHSG, α_2 -Heremans–Schmid glycoprotein; Ga(III)-IDA, gallium(III)-iminodiacetic acid.

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AHSG; in conjunction with the results presented here, this suggests that the circulating pool of AHSG is composed of two fractions, one (constituting approx. 20%) containing at least 1 mol of phosphate/mol of AHSG, and the remaining 80% containing no phosphate.

EXPERIMENTAL

Materials

Human AHSG was obtained from Sigma (catalogue no. G 0516; St Louis, MO, U.S.A.), as were protamine sulphate, 7-aminoheptanoic acid (7-AHA) and protamine-agarose (lot no. 83H9520). [^{32}P] P_i , Sephacryl 300, PD-10 columns, MiniQ ion-exchange column and CNBr-activated agarose were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Human fibrinogen grade L was obtained from Chromogenix AB (Mölnådal, Sweden). Malachite Green reagent [0.23 g/l Malachite Green/8.2 g/l ammonium heptamolybdate tetrahydrate/0.5 g/l poly(vinyl alcohol) in 1 M HCl] was obtained from Apoteksbolaget, Södersjukhuset (Stockholm, Sweden). Modified trypsin was from Promega (Madison, WI, U.S.A.) and endo-proteinase Asp-N was from Roche Diagnostics (Mannheim, Germany). All chemicals, as well as the water used, were checked before use for phosphate contamination.

Blood samples

Human cubital venous blood samples were drawn from healthy volunteers in our laboratory. ID1 and ID2 were from the same individual, a 24-year-old female; ID1 was taken in the third trimester of pregnancy and ID2 was collected 6 months after parturition. ID3 was a 52-year-old female and ID4 was a 24-year-old male. Blood was collected in EDTA Vacutainer® tubes (Becton Dickinson Vacutainer Systems Europe, Meylan, Cedex-France). After 10 min at room temperature the blood was centrifuged at 1200 *g* for 10 min at 20 °C. To 1 ml of plasma was added 10 μl of 1 M 7-AHA as anti-proteolytic agent. The plasma was aliquoted into 350 μl portions, frozen immediately and stored at -70 °C until use.

Isolation of AHSG from blood plasma

AHSG was purified by two different chromatographic methods in which the first (method 1 below) involved two steps of chromatography on two different protamine-agaroses. The first step used protamine-agarose manufactured in our laboratory and the second step was performed on commercial protamine-agarose.

In an alternative method (method 2 below) we used gel filtration on Sephacryl 300 as a first step to separate large proteins, mainly fibrinogen, from smaller ones. This was followed by a protamine-agarose purification step of the fractions containing AHSG. The purification was performed at room temperature unless stated otherwise. The eluted fractions were stored in an ice-water bath until further processing.

Method 1

In the first step the protamine-agarose column (0.5 ml) was equilibrated in 50 mM Tris/HCl/5 mM EDTA/5 mM 7-AHA (pH 7.3) (buffer A); 700 μl of plasma diluted 1:4 in this buffer was applied to the column at a rate of 100 $\mu\text{l}/\text{min}$. The column was washed with buffer A until A_{280} was less than 0.05 and then with buffer A containing 700 mM NaCl (buffer A/700). This

buffer eluted AHSG as well as other proteins. Fibrinogen was not eluted with this buffer but was subsequently eluted from the column with 50 mM Tris/HCl/5 mM EDTA/5 mM 7-AHA (pH 4.5) (buffer B). The column was discarded after use.

The buffer A/700 fraction containing protein was desalted in accordance with the manufacturer's description on a PD-10 column equilibrated and eluted with buffer A; the eluate was applied to a column (1.6 ml) containing commercially available protamine-agarose (Sigma) equilibrated in buffer A. The column was washed with buffer A until A_{280} was less than 0.05 and then with buffer A containing 240 mM NaCl (buffer A/240). AHSG was finally eluted with buffer B; the pH of the eluate was immediately raised to neutrality by the addition of 125 μl of 0.1 M Tris to 2 ml of eluate, then aliquoted into five 300 μl portions and stored at -20 °C for later phosphate analysis. Samples were also prepared for SDS/PAGE analysis and stored at -20 °C.

The column was eluted with 0.2 M sodium citrate/citric acid, pH 5.3, to release proteins not eluted by previous buffers. The column was finally washed repeatedly (four to six cycles) with 15 ml of 50 mM Tris/HCl/2 M urea (pH 4) alternating with 15 ml of 50 mM Tris/HCl/2 M NaCl (pH 8) before being used again.

Method 2

In the second purification scheme, 1 ml of plasma was subjected to gel filtration on Sephacryl 300 (19 cm \times 1.4 cm²) equilibrated in buffer A, and eluted at a flow rate of 12 ml/h; 2 ml fractions were collected. After fibrinogen and other large plasma proteins had been eluted, a major peak was observed containing proteins in the size range of approx. 40–200 kDa as determined from subsequent analysis by non-reduced SDS/PAGE. These protein fractions were pooled (6 ml) and loaded on commercial protamine-agarose, which was washed and eluted as above.

The main protein peak eluted by buffer B from the commercial protamine-agarose column was subjected to a buffer change on PD-10 to 100 mM Tris/HCl, pH 7.4 (buffer C) and loaded on a Mini-Q ion-exchange column equilibrated with this buffer. Proteins were eluted from the column by a gradient of NaCl (0–2 M) in buffer C.

Phosphate analysis

The phosphate covalently bound to AHSG was released by alkaline hydrolysis and the amount of P_i was determined on microtitre plates by a modification of the Malachite Green method [28]. Human plasma contains an average of 1 $\mu\text{mol}/\text{ml}$ non-bound P_i , but no non-protein-bound P_i from blood plasma was eluted with the purified AHSG. This was shown by adding [^{32}P] P_i to the plasma before separation on protamine-agarose. Most of the [^{32}P] P_i (more than 98%) was eluted from the protamine-agarose by buffer A; the rest was eluted by buffer A/700. No labelled phosphate was detected after the second protamine-agarose purification step even if the amount of labelled phosphate added to the plasma before the first purification step exceeded 10^6 c.p.m., corresponding to a minimum of 1 c.p.m./pmol of P_i . This was taken as proof that not even minute amounts of non-bound phosphate were associated with the AHSG fractions.

SDS/PAGE

Eluted fractions were analysed by discontinuous SDS/PAGE (10 cm \times 7 cm) under non-reduced and reduced conditions by the methods of Laemmli [30] and O'Farrell [31]. To 2 vol. of sample

was added 1 vol. of sample buffer [0.25 M Tris/HCl (pH 6.8)/36% (v/v) glycerol/6% (w/v) SDS]. For reduced gels 2-mercaptoethanol was added to the sample to a final concentration of 0.5 M and the samples were heated at 95 °C for 5 min before being loaded. Gels were stained colloiddally overnight in 0.08% Coomassie Blue G-250 in 8% (w/v) $(\text{NH}_4)_2\text{SO}_4$ /1.6% (v/v) phosphoric acid/20% (v/v) methanol [32], rinsed with 25% (v/v) methanol and destained in water.

Phosphate-to-AHSG ratio for samples ID1–ID4

This ratio was determined on plasma samples purified by method 1. The amount of phosphate was determined as described above and the amount of AHSG was estimated from densitometric scanning of SDS/PAGE gels.

Peptide mass analysis

Aliquots from MiniQ fraction 2 containing AHSG were digested in solution with trypsin or Asp-N for 3 h at 37 °C. The digest was desalted on Poros R2 beads (unless stated otherwise) essentially as described by Wilm et al. [33] directly into nanospray capillaries. Analysis was performed in a tandem electrospray mass spectrometer (Q-tof; Micromass, Manchester, U.K.). Identification of peptides was by peptide mass and/or fragment data. This was interpreted by the MassLynx suite of software.

Phosphopeptide detection

In an initial series, all spectra were screened for monoisotopic peaks differing by 80 Da. All candidate peptides were then fragmented and sequence data were obtained. Here also the more hydrophobic Poros R3 was tested for desalting before the MS analysis, but no differences were found.

At a later stage the whole tryptic digest was subjected to immobilized-metal-affinity chromatography on a gallium(III)-iminodiacetic acid [Ga(III)-IDA] column as described in [34]. The eluate was desalted on either Poros R2 or Poros R3 before analysis.

RESULTS

Four different human plasma samples, ID1–ID4, were purified by method 1 as described in the Experimental section. The buffer B fractions from the second protamine–agarose column were analysed for protein-bound phosphate; SDS/PAGE was performed under both non-reduced and reduced conditions. From the protein band patterns in these gels (results not shown) we made a tentative assignment of the phosphoprotein to a broad band with an apparent molecular mass of approx. 50–60 kDa under both non-reduced and reduced conditions. To ascertain that the protein-bound phosphate was connected with this band, we designed a different isolation procedure using gel filtration of the plasma on Sephacryl 300 as a first step followed by protamine–agarose and ion-exchange chromatography.

Pooled buffer B fractions from two identical purifications on Sephacryl 300/commercial protamine–agarose (method 2) were subjected to a buffer change to buffer C on two PD-10 columns, pooled and loaded (7 ml) on a MiniQ ion-exchange column (250 μl) equilibrated in buffer C. The flow-through contained AHSG (see below). A linear gradient of 0–2 M NaCl in buffer C was applied and UV-absorbing material was eluted between 0.1–0.4 M NaCl. No more UV-absorbing material was eluted during the remainder of the gradient.

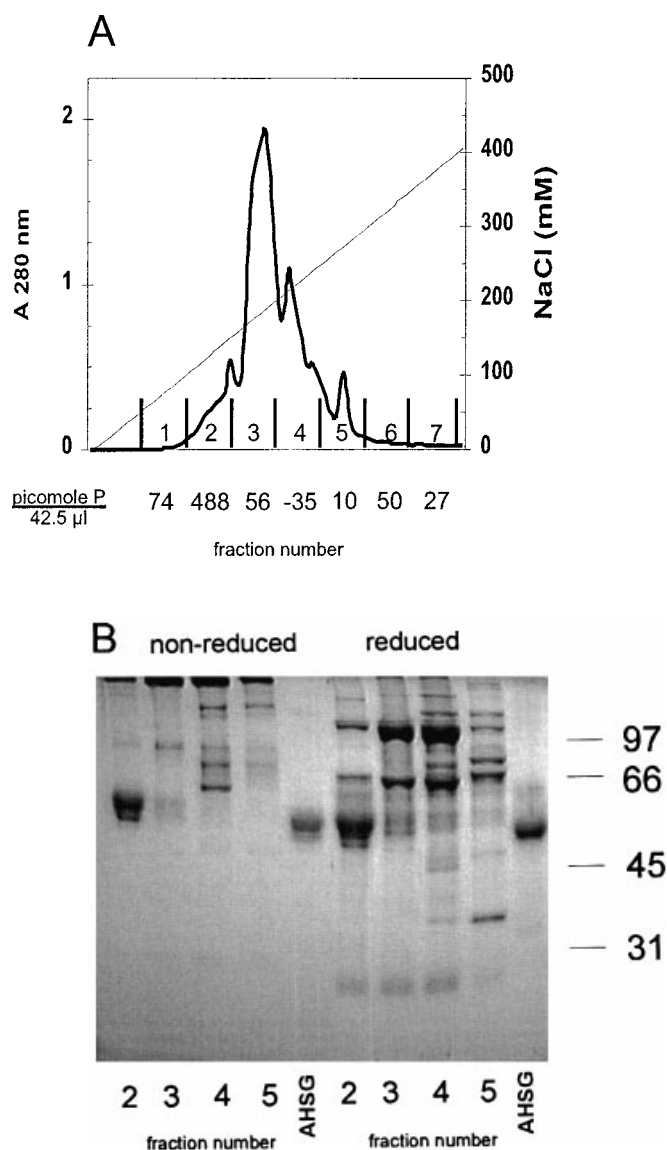


Figure 1 Ion-exchange purification of AHSG from protamine–agarose

(A) The buffer B fraction obtained from commercial protamine–agarose was fractionated on a MiniQ column in buffer C (100 mM Tris/HCl, pH 7.4) with a gradient of 0–2 M NaCl (0–0.4 M shown). The eluate was monitored at 280 nm with a cell path of 3 mm. Fraction numbers and phosphate content (pmol/42.5 μl) are also shown. The collected fraction size was 120 μl . (B) SDS/PAGE [10% (w/v) gel] of fractions 2–5 under non-reduced and reduced conditions. Fractions 2, 4 and 5 were 5 μl ; fraction 3 was 2 μl . AHSG loading was 1.35 μg . The bands were revealed by colloidal staining. The positions of molecular mass markers are indicated (in kDa) at the right.

Figure 1(A) shows the elution profile from the ion-exchange column between 0 and 0.4 M NaCl. Phosphate analysis on fractions eluted from the ion-exchange column showed that only fraction 2 contained phosphate significantly different from the inherent error of the method of analysis, approx. ± 50 pmol. Figure 1(B) shows the protein pattern in fractions 2–5 under non-reduced and reduced conditions. The concentration of AHSG in fraction 2 was estimated as 0.48 mg/ml by densitometric scanning of the bands under non-reduced conditions. Adopting a molecular mass of 44 kDa (peptide mass with added carbohydrate masses) for AHSG and a phosphate content of

Table 1 Peptides identified after trypsin digestion of human AHSG purified from plasma

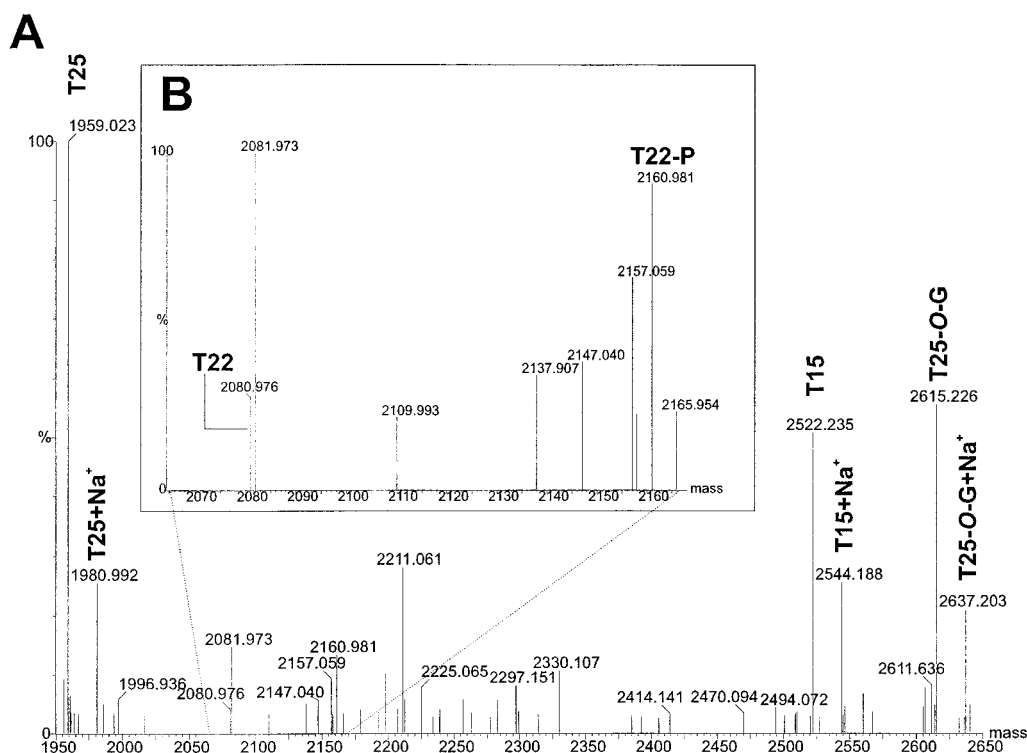
Position assignments are stated as though the protein were in the one-chain form. Abbreviation: CP, connecting peptide.

Fragment	Amino acid sequence	Position	Location
T1	APHGGLIYR	1–10	A-chain
T2	QPNCCDPETEAAALVAIDYINQNLPGWYK	11–39	A-chain
T3	HTLNQIDEVK	40–49	A-chain
T4	VWPQQPSGELFEIETLTETCHVLDPTPVAR	50–81	A-chain
T7	EHAVEGDCDFLLK	89–102	A-chain
T9	FSVYAK	107–113	A-chain
T10-P	CDSSPDS(PO ₄)AEDVR*	114–125	A-chain
T12	VCQDCPLLAPLNDTR†	127–141	A-chain
T14	AALAAFNAQNNNGSNFQLEEISR†	148–169	A-chain
T15	AQLVPLPPSTYVEFTVSGTDCVAK	170–193	A-chain
T22	HTFMGVVSLGSPS(± PO ₄)GEVSHPR	300–319	CP
T25	TVVQPSVGAAGPVVPPCPGR‡	323–343	B-chain

* Phosphopeptide isolated on Ga(III)-IDA-column.

† This peptide was solely detected in its glycosylated form.

‡ This peptide was detected in both its glycosylated and non-glycosylated forms.

**Figure 2** Part of mass spectrum of tryptic digest of fraction 2 from purification by ion-exchange chromatography

(A) Deconvoluted and declustered mass peaks ($M+H^+$) collected between 1950 and 2650 Da. T15 and T15 + Na⁺ denote the tryptic AHSG peptide T15 in the A-chain without and with associated Na⁺ respectively. T25 and T25 + Na⁺ denote the non-glycosylated tryptic AHSG peptide from the B-chain without and with associated Na⁺; T25-O-G and T25-O-G + Na⁺ denote the glycosylated tryptic AHSG peptide without and with associated Na⁺. (B) Detail of (A) between 2065 and 2165 Da. T22 and T22-P denote non-phosphorylated and phosphorylated peptides.

488 pmol/42.5 μ l, the phosphate-to-AHSG ratio was calculated as 1 mol/mol.

Tryptic digestion of fraction 2 with subsequent peptide mass analysis showed unambiguously that fraction 2 contained AHSG. Table 1 shows the peptides identified as AHSG peptides. All tryptic peptides except T10 were identified with a combination of peptide mass and sequence derived from fragmentation data, including the phosphorylated form of T22. Figure 2(A) shows

deconvoluted and declustered mass peaks as $M+H^+$ collected between 1950 and 2650 Da, whereas Figure 2(B) is a detail of the mass spectrum between 2065 and 2165 Da. Figure 3 shows fragmentation data (C-terminus to N-terminus, left to right) of peaks at $M+3H^+$ 694.34 Da (residues 319–300) and $M+3H^+$ 721.01 Da (residues 319–306), corresponding to the peaks marked T22 and T22-P in Figure 2(B), identifying serine in position 312 as being phosphorylated. The T10 peptide in its phosphorylated

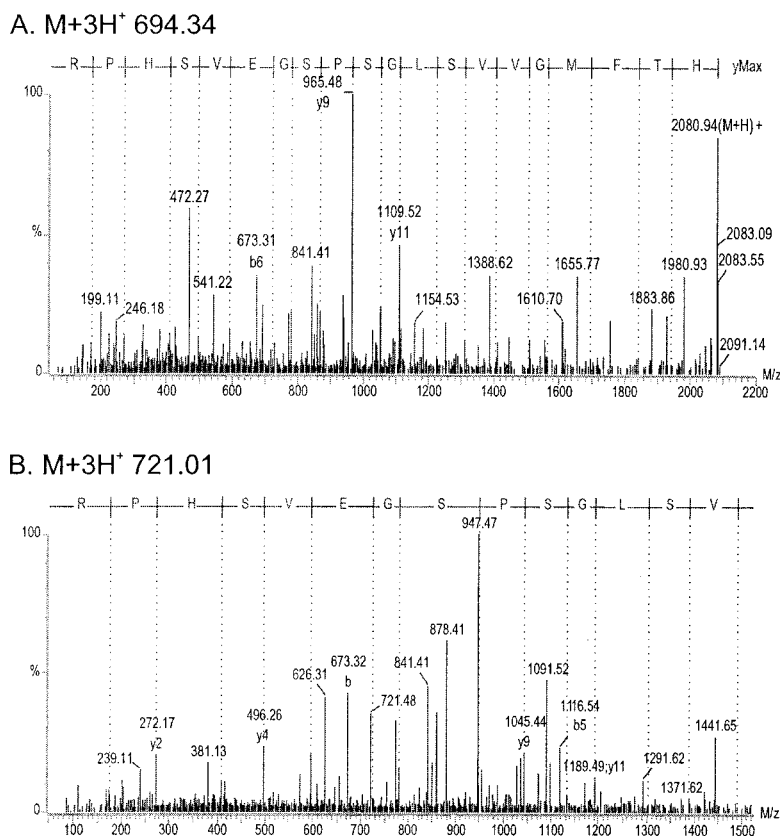


Figure 3 Fragmentation and sequencing of non-phosphorylated and phosphorylated tryptic peptide T22

(A) Fragmentation of mass peak at $M+3H^+$ 694.34 corresponding to $M+H^+$ 2080.976. The sequence reads from right to left, residues 300–319. (B) Fragmentation of mass peak at $M+3H^+$ 721.01 corresponding to $M+H^+$ 2160.981. The sequence reads from right to left, residues 306–319.

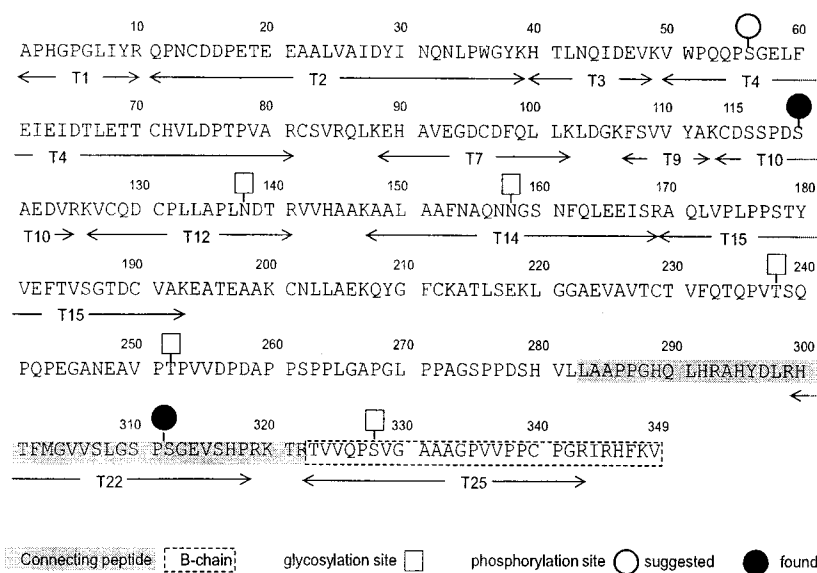


Figure 4 Amino acid sequence of human AHSR with identified and postulated post-translational modifications

Sequence and numbering correspond to the one-chain form of the protein. Arrows denote identified tryptic peptides.

Table 2 Partial sequence, monoisotopic neutral masses and position assignment

Partial sequence	Observed mass (Da) (monoisotopic)	Calculated mass (Da) (monoisotopic)	Position assignment
-PSVGAAGPVVP-	2738.63	2738.69	327–338
-TVVQPSVGAAGPVVP-	3394.93	3394.91	323–338

Table 3 Peptides identified after Asp-N digestion of human AHSG purified from plasma

Position assignments are stated as though the protein were in the one-chain form. 'D20' denotes part of the D20-peptide (residues 297–349). Abbreviation: CP, connecting peptide.

Fragment	Amino acid sequence	Position	Location
D1	APHGPGLIYRQPNC	1–14	A-chain
D4	DYINQNLPGYKHTLNQI	28–45	A-chain
D6	DTLETTCHVL	65–74	A-chain
D7	DPTPVARCSVRQLKEHAVEG	75–94	A-chain
D9	DFQLLKL	97–103	A-chain
D10	DGKFSVYAKC	104–114	A-chain
D18	DAPSPPLGAPGLPPAGSPP	258–277	A-chain
D19	DSHVLLAAPPQHQLHRAHY	278–296	A-chain
'D20'	DLRHTFMGVVSLGSPS(± PO ₃)G	297–313	CP
'D20'	DLRHTFMGVVSLGSPSGEVSHPR	297–319	CP
'D20'	DLRHTFMGVVSLGSPS(± PO ₃)GEVSHPRKT	297–321	CP
'D20'	TVVQPSVGAAGPVVPCPGRIRHFKV*	323–349	B-chain

* This fragment constitutes the complete B-chain; the peptide was detected in both its glycosylated and non-glycosylated forms.

form was isolated by immobilized-metal-affinity chromatography of the tryptic digest on a Ga(III)-IDA column and was identified as this peptide by MS and sequencing as above, revealing that serine-120 contained the phosphate group (results not shown).

Figure 4 shows the complete sequence of single-chain AHSG, including glycosylation and phosphorylation sites, together with a map of the identified tryptic peptides.

The use of dithiothreitol to reduce fraction 2 from the ion-exchange column, followed by MS analysis, resulted in two distinct peaks at $M + 4H^+$ 685.666 Da and $M + 4H^+$ 849.739 Da. These masses correspond to monoisotopic neutral masses of 2738.63 and 3394.93 Da. Table 2 shows the partial sequences determined from the two peaks together with observed and calculated monoisotopic neutral masses. The peak at 2738.63 Da contained PSVGAAGPVVP (single-letter amino acid codes), which corresponds to residues 327–338 (see Figure 4). The monoisotopic mass of the complete peptide sequence of the B-chain was calculated as 2738.69 Da. The heavier peak corresponded to the calculated mass of the B-chain O-glycosylated with the sialotrisaccharide at serine-328 reported by Gejyo et al. [35]. From the peak heights in the mass spectrum the relative distributions of non-glycosylated and glycosylated B-chain were calculated as 57% and 43% respectively (results not shown).

Digestion of fraction 2 with Asp-N gave rise to several peptides identified by MS. Table 3 shows the peptides identified, where peptide D20 should contain part of the connecting peptide and the entire B-chain (residues 297–349) if the one-chain form of the protein was present. However, our data showed only fragments of this peptide, denoted 'D20' in this paper, in which the longest fragment contained residues 297–321. In addition, the complete B-chain without or with a sugar residue was identified with similar relative distributions of non-glycosylated and glycosylated forms (55% and 45% respectively) (results not shown). This distribution corresponded to that of peptide fragment T25 containing this glycosylation site in the tryptic digest (62% and 38% respectively) (see Figure 2A).

Database searches on peptide masses together with partial sequence information (Mascot, London, U.K.) revealed that fraction 2 also contained small amounts of IgG4, molecular mass 150 kDa (subunits 50 and 25 kDa) and complement factor C3, molecular mass 180 kDa (subunits 115 and 75 kDa). These masses are in accordance with those of the weak protein bands seen in fraction 2 in Figure 1(B). Both contaminating proteins were present in much higher quantities in fractions 3 and 4; nevertheless, these fractions contained no phosphate. It was therefore regarded as safe to assume that the phosphate measured in fraction 2 was bound to AHSG. These contaminating bands were also present to different degrees in non-reduced and reduced SDS/PAGE gels of buffer B fractions obtained in the purification scheme by method 1 (results not shown). Similarly, fractions eluted after the main buffer B peak contained these contaminants but no phosphate.

The ratio between phosphorylated and non-phosphorylated T22 was estimated from peak heights in the mass spectrum normalized to masses $M + H^+$ of the tryptic digest (see Figure 2B). The relative amounts of T22-P and T22 were 77% and 23% respectively. For T10-P and T10 no ratio could be calculated because only the phosphorylated form was retained by Ga(III)-IDA; neither T10-P nor T10 was detected in the direct mass spectrum.

The degree of phosphorylation of AHSG in fraction 2 (ID4) can therefore be estimated from MS data to be higher than 0.8 (T22-P) and possibly higher than 1 (T22-P + T10-P), which was the value calculated from phosphate and concentration determinations on fraction 2.

The total amount of AHSG retained by the MiniQ ion-exchange column was approx. 57 µg as calculated from the concentration determination on fraction 2. Our estimate of the amount of AHSG loaded was at least 3-fold that amount; we therefore suspected that some AHSG could have eluted in the flow-through fraction from the column. This fraction (9.5 ml) was therefore concentrated on a protamine-agarose column.

Table 4 Ratios between protein-bound phosphate and AHSG

Samples ID1 and ID2 were from the same 24-year-old female, pregnant (ID1) and 6 months after parturition (ID2); sample ID3 was from a 52-year-old female; sample ID4 was from a 24-year-old male. Phosphate concentrations are means \pm S.D. AHSG concentrations are calculated as the densitometrically scanned gel area of AHSG per μ l of loaded sample on non-reduced SDS/PAGE.

Sample	Phosphate concentration (pmol/127.5 μ l)	AHSG concentration (pixel/ μ l)	Phosphate-to-AHSG ratio
ID1	310 \pm 29	1802	0.172
ID2	227 \pm 44	1426	0.159
ID3	313 \pm 37	1866	0.168
ID4	224 \pm 49	1284	0.174

Buffer B elution fractions did indeed contain AHSG (approx. 240 μ g as estimated from concentration determination on non-reduced SDS/PAGE) but no phosphate was detected within the limits of our phosphate analysis; neither did mass spectra nor chromatography on Ga(III)-IDA of a tryptic digest of this fraction detect any phosphorylated peptides. The sample loaded on the MiniQ column can therefore be estimated to have contained 20% phosphorylated (1 mol/mol) and 80% non-phosphorylated AHSG, resulting in an average degree of phosphorylation in the plasma pool of approx. 0.2 mol/mol.

However, the relative distribution of glycosylated and non-glycosylated T25 (in the B-chain) in the flow-through fraction remained the same as for the phosphorylated fraction from the ion-exchange column.

The relative phosphorylation ratios for AHSG from ID1–ID4 purified by method 1 are almost identical. This is shown in Table 4, where the ratios between phosphate and the scanned gel area per μ l of loaded sample are given.

DISCUSSION

We have developed a highly sensitive method for the determination of P_i in the range 100 pmol and above [28]. We are therefore able to determine protein-bound phosphate *in vivo* under normal physiological conditions without having to depend on cell experiments with the incorporation of [32 P]P $_i$ into proteins as a tool for phosphoprotein studies.

In the initial stages of our investigation of fibrinogen and its phosphorylation *in vivo* we found inexplicably high amounts of phosphate, which led us to consider the presence of other phosphorylated proteins in the eluted fibrinogen fractions from the commercial protamine-agarose. We tentatively assigned a protein with an apparent molecular mass of 50–60 kDa on SDS/PAGE run under reduced and non-reduced conditions as a putative candidate for the unknown phosphoprotein; a different isolation scheme was devised for this protein that also included ion-exchange chromatography.

Owing to the small fraction volume (120 μ l) from the ion-exchange column, the phosphate in fractions 1–7 was measured in a single microtitre plate well; it was therefore impossible to report S.D. values for this determination. In addition, owing to the high sensitivity of the method, there is always a potential risk of contamination, resulting in erroneous values. However, the blank value (buffer C) in sextuplicates was 18.8 ± 28.8 pmol/127.5 μ l. This value and the S.D. were typical for a phosphate analysis of sextuplicates; sextuplicate samples of standard fibrinogen included on the same microtitre plate gave 387.1 ± 31.2 pmol. The average result from four previous phosphate determinations on this fibrinogen sample was 393.7 pmol with a combined S.D. of 46.8 pmol; the phosphate analysis of fractions 1–7 could therefore be regarded as valid, albeit less

accurate than usual. The values for fractions 2 and 3 were confirmed in a second one-well analysis.

The quantification of AHSG on SDS/PAGE can generally be regarded as only approximate owing to the broadness of the band on the gel and contamination with IgG (reduced gels) and also the fact that AHSG is not easily stained (approx. 3–4-fold less efficiently than albumin). The concentration data corresponded to the UV absorption value on the basis of an $A_{278}^{1\%}$ of 5.6 [16], which increased the reliability of the data.

The tryptic digest of fraction 2 with subsequent peptide mass analysis showed that the identity of the protein was AHSG. Out of 19 possible tryptic peptides with masses of more than 500 Da, we have identified 11. Fragments T12 and T14 were identified as N-glycosylated peptides with an additional mono-isotopic mass of 2204.77 Da, corresponding to the calculated mass of the biantennary N-glycan described in [36]. No non-glycosylated fragments T12 and T14 were found, indicating 100% glycosylation on these sites. The T25 fragment (residues 323–343 of the B-chain) was identified in both glycosylated and non-glycosylated forms. The tryptic fragment T20 (residues 220–293) with two O-glycans, described by Watzlawick et al. [36] as identical with that of the B-chain, was not found in the spectrum, probably owing to its large molecular mass; neither was the T20 fragment devoid of glycosylation.

Two of three postulated phosphorylation sites [18] were identified. The peptide fragment T22 (residues 300–319 in the connecting peptide) was identified in the MS of the tryptic digest in both its phosphorylated (at serine-312) and non-phosphorylated forms (see Figures 2B and 3) and the peak heights in the mass spectrum indicated that the protein was phosphorylated at this site to 77%.

The second phosphorylation site at serine-120 was only identified as a tryptic peptide T10 in its phosphorylated form after affinity chromatography on Ga(III)-IDA.

The third postulated phosphorylation site at serine-56 in human AHSG [18] is located in peptide T4 in the tryptic digest. This peptide was detected in its non-phosphorylated form in the mass spectrum but the phosphorylated form of this peptide could not be detected and this site is possibly not subjected to phosphorylation. This is supported by the fact that this phosphorylation site is not present in the protein sequence determined from cDNA obtained from African green monkey, *Cercopithecus aethiops* (GenBank[®] accession number AB004046); neither is it present in most other mammalian species (see [8]), so it is thus not a conserved site. Human fetuin-B [2] does not contain any of the postulated consensus phosphorylation sites.

Digestion of fraction 2 with Asp-N resulted in several identifiable peptides. If the protein were in a one-chain form, peptide D20 in its entire length should contain part of the connecting peptide and the B-chain. We were unable to locate

this peptide in the mass spectrum, which indicated that our preparation was a two-chain form and was thus clipped at arginine-322, resulting in a truncated D20 peptide. We identified three different 'D20' fragments, the longest containing all the C-terminal residues of the heavy A-chain with only arginine-322 missing. In addition, this fragment was identified in both its non-phosphorylated and phosphorylated forms in the mass spectrum, as was 'D20', residues 297–313. The fragment containing residues 297–319 was identified in the spectrum in the non-phosphorylated form. The D-peptide containing the other identified phosphorylated site (serine-120) consisted of residues 119–122 and was probably too short to be detected in the mass spectrum.

Reduction of fraction 2 with dithiothreitol, followed by MS of the sample, showed that this released the B-chain of AHSG, revealing the presence of the heterodimeric form of AHSG. Digestion of the protein with Asp-N also implied that our preparation was a heterodimer. These facts do not completely rule out the possibility of the existence in blood of the one-chain form; nevertheless, we have so far been unable to discover it in our preparation; neither has AHSG been reported as a single-chain protein from human plasma preparations [10,17,24].

The presence of the B-chain and fragment T25 in both glycosylated and non-glycosylated forms in approximately the same relative distributions irrespective of sample treatment could indicate a biological function for this modification, although it could be a trivial result of dissociation in the mass spectrometer. Gejyo et al. [35] reported that their preparation of AHSG contained predominantly the glycosylated form of the B-chain and only minor amounts lacking the carbohydrate unit. Their AHSG was presumably prepared from pooled human plasma and thus reflects an average of the B-chain, whereas our preparation for MS was from one single individual. Because protein glycosylation is known to serve a variety of functions, these different results concerning glycosylation might well imply differences in intrinsic biological activity of the protein [37]. Thus we speculate whether the degree of glycosylation of AHSG at this site can serve as a biomarker for different pathological conditions.

The apparent fractionating of the protein on a MiniQ column can possibly be explained by the presence in plasma of an AHSG fraction (approx. 80%) containing no covalently bound phosphate that is therefore not retained by the ion-exchange column. This assumption is supported by an estimated average degree of phosphorylation of at least 0.2 mol of phosphate/mol of AHSG as measured on buffer B elution fractions purified by method 1 (results not shown). Further, rat plasma fetuin (pp63) consists of fractions both with and without covalently bound phosphate [9,15]. There have been some indications previously that phosphorylated AHSG could be present in human serum. Srinivas et al. [38] presented indirect evidence that human serum AHSG was at least partly phosphorylated by showing that AHSG isolated from human serum specifically inhibited insulin-stimulated insulin receptor autophosphorylation *in vitro* and *in vivo* as well as exogenous substrate tyrosine phosphorylation, although the effect of the serum-derived AHSG was 50–100-fold less efficient than that of the recombinant, phosphorylated AHSG [12]. Because it is a common observation that the non-phosphorylated protein is biologically inactive, the results of Srinivas et al. indicate that at least some phosphorylated AHSG was present in human plasma. Our results indicate that as much as 20% of the circulating AHSG in human plasma is phosphorylated at one or both of the two identified sites. The assumption that the AHSG plasma pool is not fully phosphorylated is further supported by Jahnen-Dechent et al. [18], who reported that AHSG secreted by HepG2 cells was only

partly phosphorylated, in both the one-chain and two-chain forms. Fetuin has been shown to be transiently phosphorylated inside rat hepatocytes [15]. Whether the whole population of AHSG (fetuin) is subjected to transient phosphorylation cannot be commented on, because no investigator has yet reported convincingly the presence of 100% phosphorylated fetuin in intact cells. Thus we cannot exclude the existence of a non-phosphorylated fraction of the protein in the cell. Dephosphorylation also occurs in rat plasma, according to the results of Le Cam et al. [15].

The fact that AHSG has now been isolated in its phosphorylated form from human plasma has far-reaching consequences. It implies that the effects seen with cell-derived and recombinant human AHSG in experiments *in vitro*, in which phosphorylation of the protein is a prerequisite, might also constitute real physiological functions of the protein *in vivo*. Mathews et al. [19] have shown recently that incubation with 1.8 μM recombinant human AHSG^{bae}, which is known to be at least partly phosphorylated, inhibited insulin-induced insulin receptor autophosphorylation by more than 80% in intact rat fibroblasts expressing the human insulin receptor. From our finding that approx. 20% of AHSG is phosphorylated in plasma and assuming a plasma AHSG concentration of 0.3–0.6 mg/ml, it can be calculated that the concentration of phosphorylated AHSG in the circulating pool is 1.4–2.8 μM ; i.e. in the range reported by Mathews et al. This suggests that the effects reported by Mathews et al. might well constitute real effects *in vivo*.

It might have been expected that the samples from the pregnant donor would have differed in degree of phosphorylation in view of the insulin resistance that occurs in pregnancy; however, from the data in Table 4 it is evident that the average degree of phosphorylation did not vary between AHSG purified from the donor in the third trimester and after her pregnancy, during which the donor experienced no complications whatsoever. It is also evident from Table 4 that there was no difference in phosphorylation ratio between any of these individuals in spite of their differences in sex, physiological status and age. These results do not discriminate between phosphorylation sites; neither do they reveal information about differences in glycosylation. We shall address the significance of the observed phosphorylation at serine-120 and serine-312 and the presence of non-glycosylated and glycosylated B-chain in future experiments.

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