Formation of the α_1-microglobulin chromophore in mammalian and insect cells: a novel post-translational mechanism?

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Abstract **α_1-Microglobulin is an immunosuppressive plasma protein synthesized by the liver. The isolated protein is yellow-brown, but the hypothetical chromophore has not yet been identified. In this work, it is shown that a human liver cell line, HepG2, grown in a completely synthetic and serum-free medium, secretes α_1-microglobulin which is also yellow-brown, suggesting a de novo synthesis of the chromophore by the cells. α_1-Microglobulin isolated from the culture medium of insect cells transfected with the gene for rat α_1-microglobulin is also yellow-brown, suggesting that the gene carries information about the chromophore. Reduction and alkylation or removal of N- or O-linked carbohydrates by glycosidase treatment did not reduce the colour intensity of the protein. An internal dodecapeptide (amino acid positions 70–81 in human α_1-microglobulin) was also yellow-brown. The latter results indicate that the chromophore is linked to the polypeptide. In conclusion, the results suggest that the α_1-microglobulin gene carries information activating a post-translational protein modification mechanism which is present in mammalian and insect cells.**

Key words: α_1-Microglobulin; Protein HC; Chromophore; Insect cell; Post-translational modification

1. Introduction

α_1-Microglobulin (α_1-m), is a 26–28 kDa plasma protein that has immunoregulatory properties [1]. It is synthesized in hepatocytes from an mRNA encoding a precursor protein, α_1-m-bikunin [2]. Bikunin is a Kunitz type proteinase inhibitor [3] which has stimulatory effects on some cells [4,5] and is essential for the building of extracellular matrix [6]. The α_1-m-bikunin precursor is cleaved intracellularly, and free α_1-m secreted from the hepatocytes [7]. In the blood, however, only about 50% of α_1-m is found in a free form, and the remaining portion is found in high molecular weight complexed forms. Thus, α_1-m-complexes with human albumin [8], human IgA [9], the rat α-macroglobulin α_1 inhibitor-3 [10], and rat fibronectin [11] have been demonstrated.

α_1-m is a member of the lipocalin superfamily, which comprises around twenty different proteins with a predicted three-dimensional structure of two antiparallel β-sheets surrounding a hydrophobic pocket [12]. Human α_1-m consists of 183 amino acids [2] and is substituted with two asparagine- and one threonine-linked oligosaccharide (abbreviated to *N*- and *O*-glycan, respectively) [13] (see also Fig. 1). α_1-m isolated from various sources is yellow-brown, but the structure and location of the hypothetical chromophore is not known. In this work, we have studied the chromophore of α_1-m isolated from several different sources, including insect cells transfected with a virus carrying the α_1-m-bikunin gene. The results suggest that information about the formation of the chromophore is carried in the α_1-m gene, that the chromophore is synthesized de novo by the cells, and that it is attached to the α_1-m polypeptide.

2. Materials and methods

2.1. HepG2 cell culture in serum-free medium

The human hepatoma cell line HepG2 was cultivated in a serum-free medium supplemented with selenium [14]. Cells were grown in 175 cm^2 flasks (Nunc, Roskilde, Denmark) with 50 ml RPMI 1640 (Gibco BRL, Gaithersburg, USA), containing 3×10^{-8} M Na_2SeO_3 (Sigma Chemical Co.), 0.3 g/l L-glutamine, 50 mg/l penicillin and 50 mg/l streptomycin, at 37°C in CO_2/air (5:95). The medium, containing 0.5–1 mg/l human α_1-m, as measured by radioimmunoassay [15], was harvested after 72 h, and the cells from each flask were trypsinated and distributed into four new flasks. NaN_3 was added to 0.3 mM, and the medium was frozen.

2.2. Expression of α_1-m in Hi 5 insect cells

Rat α_1-m-bikunin encoding cDNA [16] was ligated into the transfer vector pVL 1392 (Invitrogen, San Diego, USA) and recombined into a wild-type baculovirus (BacPAK6; Clontech, Palo Alto, USA). The resulting α_1-m-bikunin encoding baculovirus was used to infect *Trichoplusia ni* (Hi 5) insect cells (Invitrogen), grown in Ex-cell 401 medium (JRH Bioscience, Lenexa, USA). The Hi 5 cells secreted α_1-m into the medium, both in free form and as the precursor protein α_1-m-bikunin, at a total concentration of approximately 15 mg/l. The whole procedure has been described elsewhere (Bratt and Åkerström, submitted for publication).

2.3. Purification of α_1-m

Human α_1-m was purified from the urine of patients with tubular proteinuria or from HepG2 cell culture medium. The rat homologue of α_1-m was purified using rat urine from animals with kidney impairment [17] as starting material, or from the culture medium of transfected Hi 5 insect cells. The urine or cell culture medium was concentrated by ultrafiltration, and then subjected to affinity chromatography on a column containing monoclonal mouse anti-(rat and human α_1-m) antibodies BN 11.10 [18], immobilized to Affi-gel Hz (40 mg/2 ml gel) according to instructions provided by the manufacturer (Bio-Rad Laboratories). The column was equilibrated with 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 5 mM EDTA and 0.02% NaN_3. After application of the sample and washing with the Tris buffer, the column was eluted with 0.1 M glycine-HCl, pH 2.5, and the eluted fractions were immediately neutralized with 1/10 vols. 1 M Tris-HCl, pH 8.5. The eluted fractions were pooled, concentrated and applied to gel chromatography on a column packed with Sephacryl S-200 (Pharmacia AB Biotechnology, Uppsala, Sweden), equilibrated and eluted with 20 mM Tris-HCl, pH

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Abbreviations: α_1-m, α_1-microglobulin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; Endo-GalNAc, endo-*N*-acetyl-galactosaminidase; N-glycan, asparagine-linked oligosaccharide; O-glycan, threonine-linked oligosaccharide; CHO, carbohydrate; Hi 5, *Trichoplusia ni*.

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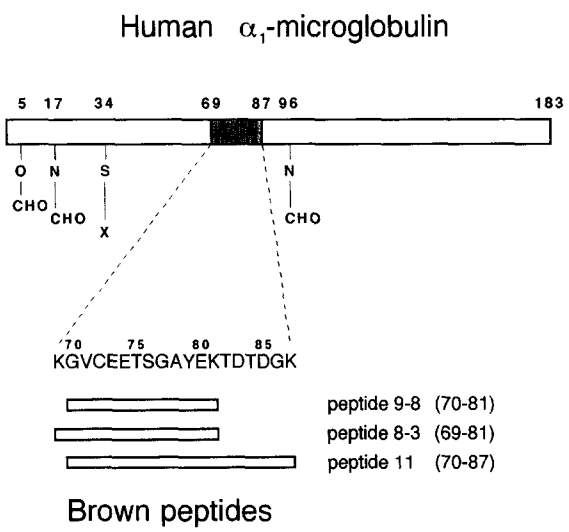

Fig. 1. Schematic representation of human α_1-m. The 183-amino acid polypeptide is substituted with one O-linked carbohydrate moiety (CHO) bound to the threonine residue at position 5 and two N-linked carbohydrate moieties bound to the asparagine residues at positions 17 and 96. The cysteine residue at position 34 is linked to an unknown substance (X). The three yellow-brown peptides 9–8, 8–3 and 11 were isolated as described in section 2.

8.0, 0.15 M NaCl, 0.02% NaN_3. Eluted fractions were analysed by UV-absorbance at 280 nm and SDS-PAGE (see below). Fractions containing pure α_1-m or α_1-m-bikunin as determined by SDS-PAGE were pooled, dialyzed against distilled, de-ionized water and freeze-dried. All steps were carried out at 4°C.

2.4. Other proteins

Rat hormone-sensitive lipase was purified from baculovirus-transfected insect cells [19] and rat albumin from human urine as described [20]. Bovine serum albumin was purchased from Sigma.

2.5. Reduction and alkylation

α_1-m was dissolved (2 mg/ml) in a buffer containing 6 M guanidine-HCl and 0.1 M Tris-HCl at pH 8.5. Dithioerythritol was added to 20 mM and the solution was incubated for 1 h at 20°C and in complete darkness. Iodoacetamide was added, and the incubation continued for 30 min at 20°C in darkness. The samples were then dialyzed exhaustively against PBS (phosphate-buffered saline: 8 mM sodium phosphate, 1.5 mM potassium phosphate, pH 7.4, 0.12 M sodium chloride, 2.7 mM potassium chloride, pH 7.4).

2.6. Removal of carbohydrate

Neuraminic acid was removed from proteins by incubation (37°C, 20 h) with agarose-insolubilized neuraminidase (from *Clostridium perfringens*, 0.6–1 U/ml gel; Sigma Chemical Co.), in 0.1 M sodium acetate, pH 5.0, using a total protein concentration of 1 mg/ml and 1 U neuraminidase/mg protein. O-Linked glycans were removed by incubating (37°C, 48 h) neuraminidase-treated proteins with endo-α-*N*-acetylgalactosaminidase (Endo-GalNAc, from *Diplococcus pneumoniae*; Sigma), in 0.1 M sodium acetate, pH 6.0, using a total protein concentration of 1 mg/ml and 0.1 U enzyme/mg protein. After digestion with neuraminidase or galactosaminidase, the released saccharides were removed by dialysis against PBS. *N*-Glycans were digested by incubating (37°C, 72 h) the proteins with glycopeptidase F (from *Flavobacterium meningosepticum*; Sigma), in 20 mM sodium phosphate, pH 7.2, 25 mM EDTA, using a total protein concentration of 10 mg/ml, starting with 4 U enzyme/mg protein and adding another 2 U/mg protein at 24 and 48 h of incubation. The incubations were performed under dialysis against 20 mM sodium phosphate, pH 7.2, 25 mM EDTA. Between 50 and 500 μg α_1-m was used for each glycosidase digestion.

The digestion of human α_1-m with glycopeptidase F was incomplete [21], and α_1-m molecules carrying *N*-glycans were separated from those devoid of *N*-glycans by lectin affinity chromatography on concanavalin A-Sepharose (Pharmacia). A 1-ml column was equilibrated with 50 mM Tris-HCl, pH 7.7, containing 1 M NaCl and 1 mM each of $MgCl_2$, $MnCl_2$ and $CaCl_2$. The sample (5–50 μl), was applied, the column was rinsed with 10 ml of the same buffer, and bound proteins finally eluted with 3 ml of the buffer containing 0.1 M α-methyl-D-mannose.

2.7. Peptide preparation

Approximately 10 nmol (250 μg) of reduced and *S*-carboxymethylated α_1-m or bovine serum albumin was dissolved in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl and digested with 2.5 mg of bovine trypsin (Sigma) overnight at 23°C. The peptides were purified by reverse-phase HPLC on a Pharmacia Smart system equipped with a Pharmacia (μRPC C2/C18, 3.2 × 3 mm) or a Brownlee (Aquapore RP-300, 220 × 2.1 mm) column, eluting with 0.1% TFA and a linear gradient from 0 to 90% CH_3CN. Fractions were collected manually based on a 220 nm analog signal from the μPeak detector (Pharmacia). The absorbancies at 220 nm, 310 nm and 352 nm were digitalized and recorded on a computer for post-run analysis. Synthetic peptides were purchased from the BM-unit, Lund University.

2.8. Sequence determination

Peptides were sequenced by Edman degradation on an Applied Biosystems 477A sequencer with on-line detection of PTH amino acids using an Applied Biosystems 120A HPLC.

2.9. SDS-PAGE

SDS-PAGE under reducing conditions was done according to Laemmli [22]. Samples were boiled for 3 min in a buffer containing SDS and 2-mercaptoethanol before the electrophoresis. Rainbow high molecular weight standards were from Amersham. After completed electrophoresis, the separated proteins were stained with Coomassie brilliant blue.

2.10. Absorbance analysis

The light absorbance of protein samples, 0.2–0.5 mg/ml in PBS (α_1-m or albumin), or 1 mg/ml in PBS, 0.2% $C_{13}E_{12}$ (a heterogenous detergent, Berol 058; from Berol Kemi AB, Stenungsund, Sweden), 50% glycerol (hormone-sensitive lipase), were measured between 250 and 450 nm in a Uvikon 930 Spectrophotometer (Kontron Instruments), subtracting the absorbance of the solvent manually.

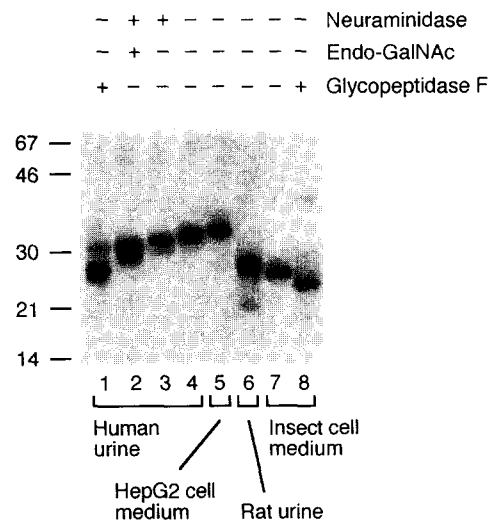

Fig. 2. Different glycosidase treatments of the human and rat α_1-m homologues isolated from various sources. Approximately 1 μg of each protein was separated by SDS-PAGE (T = 12%, C = 3.3%) in the presence of mercaptoethanol. Lane 1, human urinary α_1-m digested with glycopeptidase F; lane 2, human urinary α_1-m digested with neuraminidase and Endo-GalNAc; lane 3, human urinary α_1-m digested with neuraminidase; lane 4, non-treated human urinary α_1-m; lane 5, non-treated human α_1-m from HepG2 cell culture medium; lane 6, non-treated rat urinary α_1-m; lane 7, non-treated insect cell medium α_1-m; lane 8, insect cell medium α_1-m digested with glycopeptidase F.

3. Results

3.1. Isolation and characterization of α_1-m from different sources

α_1-m was purified from human and rat urine by anti-α_1-m affinity chromatography followed by gel chromatography on Sephacryl S-200. The purified proteins were analysed by SDS-PAGE (Fig. 2, lanes 4 and 6). Human α_1-m had an apparent molecular mass around 31 kDa and rat α_1-m consisted of two components around 28 and 20 kDa, the latter an N-terminal fragment of rat α_1-microglobulin [18]. The human liver cell line HepG2, shown previously to secrete human α_1-m [23], was cultivated in a completely synthetic medium without serum additions. Approximately 0.6 mg α_1-m could be isolated from 1 l of medium by anti-α_1-m affinity chromatography and gel chromatography. The purified protein migrated on SDS-PAGE with an apparent molecular mass around 31 kDa (Fig. 2, lane 5). The insect cell line Hi 5, transfected with baculovirus carrying DNA coding for rat α_1-m-bikunin, secreted α_1-m-bikunin and free α_1-m into the culture medium (Bratt and Åkerström, submitted for publication). Free α_1-m was purified by affinity chromatography and gel chromatography. According to SDS-PAGE, the apparent molecular mass of α_1-m from insect cell medium was 26 kDa (Fig. 2, lane 7).

All four proteins were yellow-brown as determined visually, both in freeze-dried form and in solution (not shown). The optical properties were also analysed by reading the absorbance spectrum of the solutions. A similar spectrum was seen for all α_1-m preparations (Fig. 3): a peak in the UV region was followed by a slowly declining plateau from 300 nm on to higher wavelengths. Thus, all four α_1-m preparations absorbed light both in the ultraviolet and short wavelength visible region. As a control, the absorbance spectra of rat urinary albumin (Fig. 3), and hormone-sensitive lipase, expressed by baculovirus-transfected insect cells (not shown) were determined. Both these proteins were colourless, freeze-dried or in solution, showing absorbance in the ultraviolet region only.

3.2. Deglycosylation, reduction and alkylation of α_1-m

α_1-m is a glycoprotein (see also Fig. 1). We employed glycosidases to investigate if the colour of the protein is associated with the carbohydrate. The *N*-glycans were removed by digestion with glycopeptidase F. SDS-PAGE shows that the resulting *N*-glycan-free human α_1-m has a molecular mass around 24 kDa (Fig. 2, lane 1). The *O*-glycan was removed by sequential digestion with neuraminidase and Endo-GalNAc, yielding an α_1-m species around 30 kDa (Fig. 2, lane 2). The recombinant rat α_1-m from insect cell medium has been shown to carry both *O*- and *N*-glycans (Bratt and Åkerström, submitted for publication), and these were removed by glycosidase treatment as described above. The *N*-glycan-free insect cell α_1-m was approximately 2 kDa smaller than non-treated α_1-m, as judged by SDS-PAGE (Fig. 2, lane 8). Removal of the *O*-glycan of insect cell α_1-m gave no detectable shift in apparent molecular mass (Bratt and Åkerström, submitted for publication).

By visual examination, none of the glycan-free α_1-m species showed a decreased intensity of the yellow-brown colour. The absorbance spectra showed the same slowly declining slope in

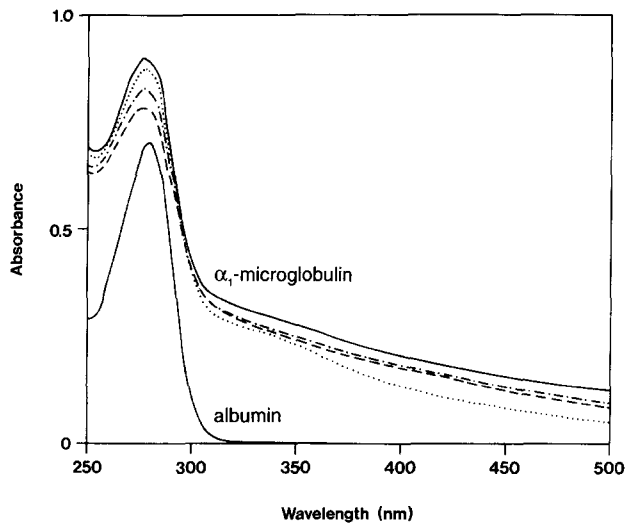

Fig. 3. Absorbance spectrum of α_1-m from various sources. The light absorbance was measured at a protein concentration of approximately 0.5 mg/ml in PBS. The human α_1-m homologue was isolated from urine (···) or HepG2 cell culture medium (—), and the rat α_1-m homologue from urine (- - -) or baculovirus-transfected insect cells (-·-·-). The lower unbroken line shows the spectrum of albumin isolated from rat urine.

Table 1
UV-light absorbance at 280 and 330 nm of various preparations of α_1-m

Protein	Source	Treatment	Absorbance (330/280 nm)
α_1-Microglobulin	Human urine	Untreated	0.31
		Reduced and alkylated	0.38
		Neuraminidase-treated	0.46
		Endo-GalNAc-treated	0.44
		Glycopeptidase F-treated	0.43
	Rat urine	Untreated	0.35
	HepG2 cell medium	Untreated	0.35
	Insect cell medium	Untreated	0.34
		Reduced and alkylated	0.45
		Endo-GalNAc-treated	0.41
		Glycopeptidase F-treated	0.35
Other proteins	Rat urine	Albumin	0.01
	Insect cell medium	Hormone-sensitive lipase	0.01

the low visible region as non-treated α_1-m. For brevity, the ratio between the absorbance at 330 nm and 280 nm, instead of the full absorbance spectra, are listed in Table 1. Evidently, the ratio was not decreased by the glycosidase treatments. The values for α_1-m, 0.31–0.46, can be contrasted to the low value, 0.01, for the control proteins, hormone-sensitive lipase and albumin, isolated from similar sources as the α_1-m molecules. Both these proteins were colourless by visual examination.

α_1-m contains an unpaired cysteine residue covalently linked to an unknown substance which has been suggested to be responsible for the colour of the protein [24]. However, reduction and alkylation of α_1-m did not reduce the intensity of the colour, as determined visually or by light absorbance at 330 nm (Table 1).

3.3. *Isolation and characterization of yellow-brown internal α_1-m peptides*

Peptides from reduced, carboxymethylated and trypsin-digested human α_1-m were separated by reverse-phase HPLC. Several peaks in the absorbance of light at 220 nm were seen in the eluate. Some of the peaks also absorbed light at 310 and 352 nm. Fractions absorbing light at all three wavelengths were re-purified by reverse-phase HPLC using different elution gradient profiles. A yellow-brown peptide (peptide 9–8) which absorbed light at 220, 310 and 352 nm was purified. Amino acid sequence analysis showed that it consisted of twelve residues, GVCEETSGAYEK, and corresponded to positions 70–81 in human α_1-m (Fig. 1). Two longer peptides were also purified (8–3 and 11), covering the same region and which were yellow-brown and absorbed light at 220, 310 and 352 nm (see Fig. 1). As a control, a trypsin digest of bovine serum albumin was run on the HPLC, the eluted peptides monitored at 220, 310 and 352 nm. As expected, no peptides derived from bovine serum albumin absorbed light at 310 or 352 nm. Finally, a synthetic peptide with the same amino acid sequence as peptide 9–8 was colourless and did not absorb light at 310 or 352 nm.

4. Discussion

The immunoregulatory plasma protein α_1-m carries a yellow-brown chromophore, the structure of which is unknown. The results in this work suggest that the α_1-m gene carries information leading to the formation of the chromophore.

α_1-Microglobulin secreted by the human hepatoma cell line HepG2 apparently carried the chromophore, since it was as intensely coloured as human urinary α_1-m and had a similar absorbance spectrum. The HepG2 cells were grown in a defined medium and none of the components of the culture medium fits the description of the chromophore, suggesting that the chromophore itself is synthesized de novo. Moreover, the medium contained no serum additions, indicating that cellular processes were responsible for the formation of the chromophore.

Insect cells, grown in a serum-free medium and transfected with baculovirus carrying the gene for α_1-m-bikunin also expressed a yellow-brown α_1-microglobulin. Thus, the mechanism leading to the formation of the chromophore is present in both mammalian and insect cells, and could possibly be a basic cellular mechanism which is present in all cells. Recently, α_1-m-bikunin mRNA was isolated from fish [25,26] and it is thus highly probable that fish α_1-m, if expressed, is also coloured yellow-brown.

Purified peptides containing amino acid residues 70–81 of human α_1-m were shown to be coloured. Previously, an unidentified substance linked to the unpaired cysteine in position 34 of human α_1-m was proposed to be identical to the chromophore [24]. It therefore seems likely that the protein carries a chromophore at several locations. These are probably situated on the polypeptide part of α_1-m, since the results in this work indicated that the carbohydrate is not coloured. α_1-m is produced as the N-terminal half of the precursor α_1-m-bikunin. The bikunin part of α_1-m-bikunin expressed by the insect cells in this work was colourless, whereas the precursor was coloured (Bratt et al., manuscript in preparation). To our knowledge, bikunin isolated from various sources has not been reported to be coloured. Thus, the chromophore is apparently formed selectively on the α_1-m part of the α_1-m-bikunin precursor. This indicates that the mechanism for chromophore formation is highly specific for α_1-m.

The structure of the chromophore is unknown. However, this and other reports (i.e. [24]) suggest that it is optically and electrophoretically heterogeneous. It is most likely small, since the total molecular mass of α_1-m carrying the chromophore is close to the calculated sum of its constituents [16,21,27]. Moreover, as discussed above, there are probably multiple chromophores on each α_1-m molecule. It is therefore tempting to speculate that several chromophore structures exist, each at separate locations and each binding to only a small percentage of the α_1-m population, explaining the heterogeneity, the small size and the multiple locations. The delineation of the detailed structure of the chromophore(s) will hopefully answer these questions, and perhaps also provide information about its function and formation.

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