α_1 -Microglobulin chromophores are located to three lysine residues semiburied in the lipocalin pocket and associated with a novel lipophilic compound

TORD BERGGÅRD,^{1,2} ARIEH COHEN,³ PER PERSSON,³ ANNIKA LINDQVIST,¹
TOMMY CEDERVALL,¹ MARIA SILOW,⁴ IDA B. THØGERSEN,²
JAN-ÅKE JÖNSSON,³ JAN J. ENGHILD,² AND BO ÅKERSTRÖM¹

¹Section for Molecular Signaling, Department of Cell and Molecular Biology, Lund University, P.O. Box 94, S-22100 Lund, Sweden

²Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710

³Department of Analytical Chemistry, Lund University, P.O. Box 124, S-221 00 Lund, Sweden

⁴Department of Biochemistry, Lund University, P.O. Box 124, S-221 00 Lund, Sweden

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Abstract

α_1 -Microglobulin (α_1 m) is an electrophoretically heterogeneous plasma protein. It belongs to the lipocalin superfamily, a group of proteins with a three-dimensional (3D) structure that forms an internal hydrophobic ligand-binding pocket. α_1 m carries a covalently linked unidentified chromophore that gives the protein a characteristic brown color and extremely heterogeneous optical properties. Twenty-one different colored tryptic peptides corresponding to residues 88–94, 118–121, and 122–134 of human α_1 m were purified. In these peptides, the side chains of Lys92, Lys118, and Lys130 carried size heterogeneous, covalently attached, unidentified chromophores with molecular masses between 122 and 282 atomic mass units (amu). In addition, a previously unknown uncolored lipophilic 282 amu compound was found strongly, but noncovalently associated with the colored peptides. Uncolored tryptic peptides containing the same Lys residues were also purified. These peptides did not carry any additional mass (i.e., chromophore) suggesting that only a fraction of the Lys92, Lys118, and Lys130 are modified. The results can explain the size, charge, and optical heterogeneity of α_1 m. A 3D model of α_1 m, based on the structure of rat epididymal retinoic acid-binding protein (ERABP), suggests that Lys92, Lys118, and Lys130 are semiburied near the entrance of the lipocalin pocket. This was supported by the fluorescence spectra of α_1 m under native and denatured conditions, which indicated that the chromophores are buried, or semiburied, in the interior of the protein. In human plasma, approximately 50% of α_1 m is complex bound to IgA. Only the free α_1 m carried colored groups, whereas α_1 m linked to IgA was uncolored.

Keywords: α_1 -microglobulin (α_1 m); chromophore; lipocalin; lysine modification; protein HC; structure

α_1 -Microglobulin (α_1 m), a charge-heterogeneous glycoprotein with immunoregulatory properties (Ekström et al., 1975; Tejler & Grubb, 1976; Ekström & Berggård, 1977; Åkerström & Lögdberg, 1990), is a member of the lipocalin protein superfamily. The lipocalins constitute a diverse family characterized by a three-dimensional (3D) structure of two antiparallel β -sheets surrounding a hydrophobic pocket. Despite the common lipocalin fold, only about 20%

amino acid sequence identity exists between lipocalins with known structure. Many members of the family have been shown to act as transport-proteins transferring small hydrophobic molecules, for example, retinoids, arachidonic acid, and various steroids, between cells (for a review, see Flower, 1996).

α_1 m is synthesized as a precursor-protein together with bikunin, a member of the pancreatic trypsin inhibitor (Kunitz) family (for a review, see Salier, 1990). The precursor-protein is cleaved in the latter part of the biosynthetic pathway and free α_1 m is secreted (Bratt et al., 1993; Thøgersen & Enghild, 1995). The bikunin part is bound to other polypeptides, forming the mature plasma proteinase inhibitors inter- α -inhibitor, pre- α -inhibitor, and heavy chain 2/bikunin before secretion (Enghild et al., 1989). The co-expression of α_1 m and bikunin has been conserved from fish to man (Hanley & Powell, 1994; Leaver et al., 1994), suggesting that the two

Reprint requests to: Bo Åkerström, Department of Cell and Molecular Biology, P.O. Box 94, Lund University, S-221 00 Lund, Sweden; e-mail: bo.akerstrom@medkem.lu.se.

Abbreviations: α_1 m, α_1 -microglobulin; PBS, phosphate-buffered saline; m/z , mass/charge ratio; amu, atomic mass unit; ES-MS, electrospray ionization mass spectrometry; TFA, trifluoroacetic acid; RP-HPLC, reversed-phase high-performance liquid chromatography.

proteins are involved in common processes. However, so far, no functional connection has been found between the two mature plasma proteins.

In plasma, more than 50% of $\alpha_1\text{m}$ is found as high molecular weight complexes with IgA, prothrombin, and albumin in man (Grubb et al., 1983; Tejler & Grubb, 1976; Berggård et al., 1997) and α_1 -inhibitor-3 and fibronectin in rat (Falkenberg et al., 1990, 1994). Neither the sites of formation nor the function of the $\alpha_1\text{m}$ complexes are known.

Purified $\alpha_1\text{m}$ from various sources is yellow-brown. Insect cells transfected with a virus carrying $\alpha_1\text{m}$ -encoding DNA also express yellow-brown $\alpha_1\text{m}$, indicating that the gene carries information leading to the formation of the chromophore. Moreover, hepatoma cells cultured in a completely synthetic medium secrete a colored $\alpha_1\text{m}$ protein, suggesting *de novo* synthesis of the chromophore. Apparently, the mechanism leading to the formation of the chromophore is present in both mammalian and insect cells, and it has been speculated that this may be a basic post-translational mechanism present in all cells (Åkerström et al., 1995).

A previous report suggested that a chromophore is associated with Cys34 of $\alpha_1\text{m}$ (Escribano et al., 1991). In this work, we show that several heterogeneous yellow-brown chromophores are also bound to Lys92, Lys118, and Lys130 of human $\alpha_1\text{m}$. Purification of different colored peptides, followed by mass analysis, showed that chromophores with masses between 122 and 282 Da were covalently attached to the three lysine residues. A 3D model, supported by fluorescence data, suggests that these residues are semiburied in the lipocalin pocket of the protein. The results also indicate that an uncolored lipophilic, hydroxylated, and aminated substance is strongly, but noncovalently associated with the chromophores.

Results

Purification and characterization of colored peptides

The $\alpha_1\text{m}$ chromophore can be identified by its absorbance at wavelengths above 300 nm. Reduced, carboxymethylated, and trypsin-digested $\alpha_1\text{m}$ was subjected to reversed-phase high-performance liquid chromatography (RP-HPLC) (Fig. 1). Peptides containing the chromophore were identified by absorbance at 350 nm and uncolored peptides by absorbance at 220 nm only. Several homogeneous peaks absorbing at 220 nm were seen in the eluate. The peptides with light absorbance at 350 nm behaved heterogeneously, suggesting variations in the structure. Six peaks with absorbance at 350 nm were collected and yellow-brown peptides in each of the pools further purified by repeated RP-HPLC (not shown). Due to the heterogeneity of the colored peptides, as many as six different purification steps were sometimes needed to obtain a homogeneous colored peptide. These were identified by Edman degradation as F₈₈LYH(K)SK, (K)₁₁₈FSR, and H₁₂₂HGPTITA(K)LYGR (Fig. 1). No amino acid could be identified in the cycles corresponding to the Lys residues in position 92, 118, and 130. These Lys residues are therefore shown within parentheses. Moreover, the peptides were not susceptible to cleavage by trypsin at these Lys residues, suggesting that they are modified and thus not recognized by the enzyme. Three of the original peaks absorbing at 350 nm apparently contained cross-linked or associated forms of the three colored peptides. Thus, one peak contained the peptides (K)₁₁₈FSR and F₈₈LYH(K)SK, a second contained H₁₂₂HGPTITA(K)LYGR and (K)₁₁₈FSR, and a third contained H₁₂₂HGPTITA(K)LYGR, F₈₈LYH(K)SK, and (K)₁₁₈FSR (Fig. 1). These peptides were re-purified on several RP-HPLC col-

Fig. 1. Analytical RP-HPLC of a tryptic digest of $\alpha_1\text{m}$. Approximately 150 μg of reduced and carboxymethylated $\alpha_1\text{m}$ was digested by trypsin and subjected to RP-HPLC. The absorbances at 220 nm (dotted line) and 350 nm (straight line) are shown. Fractions were collected manually based on the absorbance at 350 nm and colored peptides were further purified by RP-HPLC and subjected to Edman degradation. The amino acid sequences of the purified chromophore-carrying peptides are shown in the figure. The Lys residues within brackets were not recognized by Edman degradation. In the preparative runs, larger amounts (4 mg) of tryptic $\alpha_1\text{m}$ peptides were used for purification of colored peptides (see Materials and methods).

umns, but could not be separated from each other. Attempts were made to isolate colored peptides from a wide and undefined peak of colored material eluting near the end of the gradient, but no homogeneous peptides could be purified from this peak.

To identify other potentially modified peptides, the tryptic digest of α_1 m was separated by size exclusion chromatography. Ten different fractions were pooled from the size exclusion chromatography and then subjected to RP-HPLC (Fig. 2). A large part of the chromophoric peptides were eluted in the first pool, e.g., as large molecules. Calibration of the column showed that this extremely heterogeneous material did not represent nondigested α_1 m and could therefore be aggregated colored peptides. Further purification of pools 1–4 by RP-HPLC gave no distinct peaks, and no homogeneous peptides could be purified from this pool. The identity of the colored material in pools 1–4 could therefore not be determined. Pools 5–11 contained several distinct peaks absorbing at 350 nm after RP-HPLC. These were further purified by repeated RP-HPLC and identified by Edman degradation. Altogether, 21 colored peptides were purified and sequenced (see Figs. 1, 2), and all these consisted of F₈₈LYH(K)SK, H₁₂₂HGPTITA(K)LYGR, (K)₁₁₈FSR, or aggregated or cross-linked combinations of these three peptides.

Mass analysis of colored α_1 m peptides

As shown above, colored α_1 m peptides with the same sequence eluted at several positions upon size exclusion chromatography and upon RP-HPLC. An explanation for this may be that structurally different chromophores are attached to these peptides, resulting in different adsorption effects and variations in shape of the peptides. To investigate this, some of the purified peptides were subjected to ES-MS (see Table 1). The masses of two peptides with the sequence F₈₈LYH(K)SK were determined to 1,205 and 1,045 amu by ES-MS, respectively. Thus, the masses of the chromophores linked to these two identical peptides were different, 282 and 122 amu, respectively. The masses of two other purified colored peptides H₁₂₂HGPTITA(K)LYGR and (K)₁₁₈FSR were determined to 1,656 and 658 amu, respectively, by ES-MS. The data were consistent with a delta increment of 206 amu of the peptide sequence HHGPTITAKLYGR and 122 amu of the peptide sequence KFSR. The results thus suggest that the chromophores linked to Lys92, Lys118, and Lys130 are size heterogeneous.

Some of the colored peptides were further analyzed by MS/MS. A doubly charged ion at m/z 829 in the ES-MS spectrum of H₁₂₂HGPTITA(K)LYGR (a mass increment of 206 amu of the

Fig. 2. Purification of colored tryptic peptides by size exclusion chromatography followed by RP-HPLC. Reduced, carboxymethylated, and trypsin-digested α_1 m was subjected to size exclusion chromatography and 11 different fractions were pooled (top figure). Chromophore containing peptides were then purified from pools 5, 7, 8, 9, and 11 by RP-HPLC according to the absorbance at 350 nm and identified by Edman degradation (shown below). The Lys residues within brackets were not recognized by Edman degradation.

Table 1. Analysis of tryptic peptides of α_{1m} by mass spectrometry^a

Residue	Sequence	Molecular mass (Da)		
		Expected ^b	Measured	Difference
Uncolored peptides				
21–24	IYGK	479.58	479.4	0.2
25–38	WYNLAIGSTCPWLK	1,709.99	1,710.2	0.2
40–43	IMDR	533.64	533.3	0.3
44–66	MTVSTLVLGEGATEAEISMTSTR	2,384.69	2,384.2	0.5
67–68	WR	360.42	360.4	0
70–81	GVCEETSGAYEK	1,330.4	1,329.8	0.6
82–87	TDTDGK	635.63	635.2	0.4
88–92	FLYHK	706.84	706.4	0.4
93–94	SK	233.27	233.4	0.1
119–121	FSR	408.46	408.4	0.1
122–130	HHGPTITAK	961.09	960.4	0.7
131–134	LYGR	507.59	507.4	0.2
135–139	APQLR	583.69	583.4	0.3
140–147	ETLLQDFR	1,021.14	1,020.4	0.7
148–166	VVAQGVGIPEDSIFTMADR	2,005.27	2,005.4	0.1
167–183	GECVPGEQEPELIPR	1,921.15	1,920.2	0.9
Undetected peptides				
1–20	GPVPTPDNIQVQENFNISR	c	—	
95–117	WNITMESYVVHTNYDEYAIFLTK	c	—	
39	K	146.19	d	
69	K	146.19	d	
118	K	146.19	d	
Colored peptides ^c				
88–94	FLYH(K)SK	922.7	1,204.8	282
88–94	FLYH(K)SK	922.7	1,045	122
118–121	(K)FSR	536.3	658.3	122
118–121	(K)FSR	536.3	658.1	122
122–134	HHGPTITA(K)LYGR	1,450.4	1,656.4	206

^aMass spectrometry peptide mapping of α_{1m} . Human α_{1m} was digested with trypsin and the digest was subjected to ES-MS as described in Materials and methods.

^bCalculated masses were derived from the amino acid sequence of the peptides.

^cThe peptide is expected to be substituted with carbohydrates.

^dThe peptide was not in the scan range.

^eColored peptides were purified as described in Materials and methods. The modified Lys residues are displayed within parentheses.

unmodified peptide) showed strong losses of 80 and 98 amu (Fig. 3). The loss of 80 amu was the predominant cleavage and the remaining 126 amu mass increment was clearly more tightly bound to the peptide. Evidence for this comes from the y'' fragment ion at m/z 1,304 and its corresponding b2 fragment ion at m/z 275. MS/MS of the 1,205 amu ion of F₈₈LYH(K)SK (a mass increment of 282 amu) yielded a loss of 44 to 1,161 amu, and MS/MS fragmentation of the daughter ion at 1,161 amu resulted in masses of 1,046 (dominant ion, loss of 115) and 1,072 (loss of 89 amu). Fragmentation of the 1,046 ion resulted in a very stable ion at 1,029 amu, which corresponds to a mass increment of 106 amu. The mass increment, 122, of (K)₁₁₈FSR, finally, was tightly bound to the peptide and could not be fragmented. No chemical structures of the chromophores could be suggested from these data. However, the results confirm the heterogeneity of the chromophores.

All investigated colored peptides also showed a peak at 282 amu. This peak was subjected to MS/MS analysis. Virtually identical spectra were obtained from the various samples (Fig. 4). Based on the fragmentation pattern, a structure of the 282 amu

compound could be suggested (Fig. 4, insert). The two largest peaks, 261 and 246, indicate a sequential loss of a primary amine and a hydroxyl group. A series of masses ranging from 81 to 205 indicate a 12-carbon aliphatic chain. High resolution MS indicated an elementary composition of C₁₈H₃₆NO, and the smallest fragment, 81, is then compatible with cyclohexene. The position of the double bond in the cyclohexene ring is arbitrary. The cyclohexene, amine-, and hydroxyl groups are bound to the aliphatic chain separately. Although it is not possible to locate the positions of the amine- and hydroxyl groups on the aliphatic chain from the data available, it is suggested that both groups are bound to the C2 carbon due to the loss of 12 amu, as opposed to 14 amu, between masses 95 and 107. Most likely, the proposed structure does not absorb light above 300 nm and consequently is not a chromophore.

Mass spectrometry peptide mapping of α_{1m}

The purified colored tryptic peptides were obtained in a relatively low yield based on the results of the Edman degradation. The low

Fig. 3. MS/MS spectrum of a doubly charged ion at m/z 829 from a purified colored peptide with the sequence HHGPTITA(K)LYGR. A peptide absorbing at 350 nm was purified as described in the caption to Figure 1, and the amino acid sequence was determined to HHGPTITA(K)LYGR by Edman degradation. The Lys residue within brackets was not recognized by Edman degradation. ES-MS of this peptide showed a doubly charged ion at m/z 829, consistent with a delta increment of 206/207 amu of the peptide sequence given. The MS-MS spectrum of the doubly charged ion at m/z 829 is shown in the figure. The analysis was performed at M-Scan Inc., West Chester, Pennsylvania.

yield may be explained partly by the fact that each of the above-mentioned Lys residues carry size heterogeneous chromophores. In addition, the colored peptides seem to have a tendency to aggregate and form complexes as suggested above. Another factor that could potentially contribute is if only a fraction of Lys92, Lys118, Lys130 were modified. To investigate this possibility, we examined all peptides in a tryptic digest of α_1 m by ES-MS. All of the expected peptides, including the cleaved peptides F₈₈LYHK and S₉₃K, K₁₁₈, and F₁₁₉SR, and H₁₂₂HGPTITAK, and L₁₃₁YGR, were detected according to their expected m/z ratio (Table 1). This shows that only a fraction of Lys92, Lys118, Lys130 are modified. The mass of the peptide W₂₅YNLAIGSTCPWLK was 1,710 Da, consistent with the presence of carboxymethylated Cys34 in the peptide, resulting from reduction and alkylation of the native pro-

tein. Cys34 was proposed to be the major binding site between the chromophore and α_1 m (Escribano et al., 1991). It is not likely that a chromophore bound to Cys34 have been lost during reduction and alkylation, since the bond between the chromophore and α_1 m has proven to be reduction resistant. This indicates that at least part of the unpaired Cys34 residue is unmodified in the native protein. Two tryptic peptides corresponding to position 1–20 and 95–117 of the α_1 m polypeptide were not identified, probably since these peptides contain carbohydrates bound to the Thr5, Asn17, and Asn96 (Escribano et al., 1990), and therefore had molecular masses outside the scan range used in this study.

Glycation of α_1 m

The possibility of glycation of α_1 m was investigated. α_1 m was incubated with [¹⁴C]-ribose, [¹⁴C]-fructose, [³H]-galactose, or [³H]-glucose for 0–5 days. The amounts of [¹⁴C] or [³H] incorporated into α_1 m was similar to those of orosomucoid and BSA, indicating a similar glycation rate of the three proteins (not shown). The absorbance spectrum of α_1 m, which normally shows a heterogeneous absorbance at 300–400 nm, was not significantly altered after incubation with 0.5 M glucose or fructose for 0–5 days, contra-indicating a fast and specific formation of chromophores with glucose or fructose as a precursor under these circumstances. Similar amounts (1–5%), before or after trypsin digestion, of α_1 m and human serum albumin was bound to a boronate affinity column, whereas higher amounts of human serum albumin incubated in 250 mmol glucose for 30 days was bound (40–60%). The results indicate that α_1 m does not contain elevated amounts of *cis*-diol groups, which is the binding specificity of the boronate affinity gel. In conclusion, no indications of enhanced glycation or the presence of glycation products were found, suggesting novel mechanisms for the formation of the α_1 m chromophore.

Three-dimensional model of α_1 m

Based on primary and secondary structure homology, α_1 m is a member of the lipocalin family of proteins. The cup-shaped 3D structure of the lipocalins forms a pocket, which provides a binding site for various small hydrophobic molecules. A 3D model of α_1 m was obtained by alignment with the structure of the lipocalin epididymal retinoic acid-binding protein (ERABP), which was shown to be most similar to α_1 m among the lipocalins on the basis of primary structure and secondary structure predictions (Sansom et al., 1994). Figure 5 shows the model derived using the sequence of bovine α_1 m and the crystal structure of rat ERABP. Similar results were obtained using the sequence of human α_1 m. The three chromophore-carrying Lys residues are highlighted. According to this model, Lys118, Lys130, and Lys92 are semi-buried close to the entrance of the pocket.

Fluorescence spectrum of α_1 m

To verify the results from the molecular modeling of α_1 m, we investigated the fluorescence spectra of the chromophores before and after reduction and denaturation of α_1 m. The excitation wavelength, 360 nm, was chosen after obtaining excitation spectra at various emission wavelengths (not shown). Figure 6 shows that the chromophore fluorophores have a red-shift in the maximum fluorescence from 464 to 482 nm after reduction and denaturation of the protein. The results indicate a change in the immediate envi-

Fig. 4. MS/MS spectrum of an m/z 282 ion found in the purified chromophore peptides. The sample was introduced via a nano spray interface of a Bruker Esquire-LC ion trap mass spectrometer, operated in positive ionisation mode. A proposed molecular structure based on the MS/MS spectrum is inserted.

Fig. 5. Cartoon representation of a 3D model of bovine $\alpha_1\text{m}$. The model was generated using Swiss-Pdb Viewer and Swiss-Model by alignment with rat epididymal retinoic acid-binding protein retrieved as Brookhaven PDB file (1EPA). Lys92, Lys118, and Lys130 are shown in space-filling format. **A:** Viewed from the side of the β -strand barrel. **B:** Looking into the pocket.

ronment of the chromophore from a hydrophobic to a polar environment, i.e., that the chromophore is buried or semi-buried in the interior of the protein and not exposed to solvent in the native state. We also noted that the fluorescence intensity increased after denaturation. This is often seen in proteins in which chromophores are quenched by adjacent groups in the native state.

Optical properties of free $\alpha_1\text{m}$ and IgA/ $\alpha_1\text{m}$

Approximately 50% of $\alpha_1\text{m}$ is covalently linked to human plasma IgA via Cys34 and the penultimate cysteine residue of the carboxyl-terminal part of one of the IgA heavy chains by a reduction-resistant bond (Calero et al., 1994). The absorbance spectrum of IgA/ $\alpha_1\text{m}$ suggested that the complex was uncolored, in contrast to free $\alpha_1\text{m}$ (not shown). The $\alpha_1\text{m}$ part of the IgA/ $\alpha_1\text{m}$ complex was released by mild pepsin digestion and purified by Sephacryl S-300 chromatography, using conditions shown previously to yield intact $\alpha_1\text{m}$ (Calero et al., 1994). Edman degradation of the cleaved $\alpha_1\text{m}$

gave the sequences GPVP(T)PPDNI, identical to the N-terminal sequence of $\alpha_1\text{m}$, and MAEVDGT(C)Y and AEVDGT(C)Y corresponding to C-terminal nona- and octa-peptides of IgA. The MAEVDGT(C)Y sequence was the quantitatively dominating sequence. Thus, $\alpha_1\text{m}$ linked to a C-terminal IgA nona-fragment was purified and henceforth termed p9- $\alpha_1\text{m}$. Both p9- $\alpha_1\text{m}$ and urinary $\alpha_1\text{m}$ had an apparent molecular mass of 33 kDa upon SDS/PAGE under nonreducing conditions (Fig. 7). The absorbance spectra of $\alpha_1\text{m}$ and p9- $\alpha_1\text{m}$ were compared (Fig. 8). Free $\alpha_1\text{m}$ displayed the expected characteristic spectrum: a peak at 277 nm, followed by slowly declining absorbances onto higher wavelengths, whereas

Fig. 6. Fluorescence spectra of native or reduced and denatured $\alpha_1\text{m}$. 36.4 μM $\alpha_1\text{m}$ in 50 mM Mes with a pH of 6.3 was either untreated or reduced and denatured with 2.6 mM Mesna and 9 M urea. The samples were incubated 1 h at 37°C prior to analysis. Fluorescence spectra were obtained with a Perkin Elmer LB50 Fluorescence spectrometer. Excitation was at 360 nm for the unknown chromophore. The excitation slit width was 5 nm, the emission slit width was 3 nm, and the scan speed was 150 nm/min.

Fig. 7. Comparison of p9- $\alpha_1\text{m}$ and $\alpha_1\text{m}$ purified from human urine. P9- $\alpha_1\text{m}$, purified as described in Material and methods, and free $\alpha_1\text{m}$ isolated from human urine, was subjected to SDS-PAGE under nonreducing and denaturing conditions. The gel density was $T = 12\%$ and $C = 3.75\%$.

Fig. 8. Absorbance spectrum of free α_1 m and the α_1 m component of the IgA/ α_1 m complex. IgA/ α_1 m purified from human plasma was digested with pepsin. The α_1 m component of the IgA/ α_1 m complex linked to a nonapeptide of IgA (p9- α_1 m) was then purified by size-exclusion chromatography. Free human α_1 m was isolated from human urine. The light absorbances of the two proteins were measured at a concentration of ~ 0.6 mg/mL.

p9- α_1 m did not display any absorbance at wavelengths above 300 nm. α_1 m and p9- α_1 m were reduced, carboxymethylated, and digested with trypsin and subjected to RP-HPLC (Fig. 9). The elution patterns of peptides absorbing at 220 nm were identical, except that a double peak in the p9- α_1 m digest (retention time 31.5 to 32.5 min) was much smaller in the α_1 m digest. This peak was subjected to Edman degradation and shown to contain the IgA octa-peptide as well as the peptide W₂₅YNLAIGST(C)PWLK of α_1 m, i.e., containing the cross-link between IgA and α_1 m. The remaining peaks absorbing at 220 nm were similar, suggesting that p9- α_1 m is intact. In contrast, the elution pattern of peptides absorbing at 350 nm was strikingly different: no colored peptides were present in the p9- α_1 m tryptic digest. These data suggest that the α_1 m subpopulation linked to IgA does not carry the colored groups, in contrast to free, monomeric α_1 m.

Discussion

The yellow-brown color of α_1 m is conserved during evolution, suggesting that it is important to the function of the protein. All α_1 m-homologues purified to date are colored. Recently, fish α_1 m was purified from plaice liver and even this homologue carried the yellow-brown chromophores (Lindqvist & Åkerström, 1999). In this paper, we demonstrate that three different Lys-residues in α_1 m, Lys118, Lys130, and Lys92, probably closely spaced within the hydrophobic pocket of the protein, are bound to structurally heterogeneous chromophores by covalent bonds. Alignment of the amino acid sequences for the α_1 m/bikunin precursor from different species reveals that Lys118, Lys130, and Lys92 are conserved, the former two in all species including frogs and fish (Leaver et al., 1994; Hanley et al., 1995), indicating that the positions of the chromophores within the primary structure of the protein are important.

Peptide mapping of tryptic α_1 m identified all the expected peptides. This included the cleaved variants of the colored peptides, all of which were shown to have the same molecular masses as predicted from their amino acid sequence. Most likely, this is not due

Fig. 9. RP-HPLC of a tryptic digest of free α_1 m and p9- α_1 m. P9- α_1 m, purified as described in Materials and methods, or free α_1 m isolated from human urine, was reduced and carboxymethylated, and digested with trypsin, and subjected to RP-HPLC eluted with a linear 0–90% gradient of acetonitrile in 0.1% TFA. The absorbances at 220 and 350 nm are shown.

to dissociation of chromophores from α_1 m during the purification procedure, since attempts to dissociate the chromophores with 6 M guanidine-hydrochloride, SDS, acids, or bases have been unsuccessful (Tejler & Grubb, 1976; Ekström & Berggård, 1977). Thus, an unknown portion of Lys118, Lys130, and Lys92 is unmodified and not attached to chromophores. Furthermore, our data suggest that several different chromophore structures exist, each of them having slightly different chemical composition. This is supported by the extremely heterogeneous absorbance spectrum of α_1 m, indicating the presence of several colored groups in the protein. Thus, a set of structurally different chromophores attached to only a fraction of three different Lys residues can explain the observed charge and size heterogeneity of the protein.

The structures of the chromophores were not identified in this work. The low yield, picomole amounts of pure chromophore peptides from as much as 50 mg α_1 m, made interpretation of the mass spectrometry fragmentation analysis difficult. Two reasons for the low yield were discussed above: heterogeneity among the chromophores and linkage to only a fraction of the three lysine residues. In addition, the results indicate that a large part of the colored tryptic peptides are cross-linked, probably via the chromophore, further adding to the low yield. The masses of the chromophores could be determined on several of the purified colored peptides, but determination of their exact structures awaits purification of larger amounts.

A lipophilic 282-amu substance was found associated with the colored peptides. The MS/MS spectrum suggested a structure consisting of a 12-carbon aliphatic chain substituted with a cyclohexene, a hydroxyl, and an amine group. To our knowledge, this structure is previously unknown in a biological context. The substance was seen as a separate peak after MS analysis of the purified peptides, suggesting a noncovalent association. On the other hand, the association must be strong since the two components were co-purified after as much as six cycles of HPLC chromatography. Such a strong, noncovalent interaction between the colored peptides and a 282-amu lipophilic substance could be a further explanation to the heterogeneous behavior of the chromophoric peptides during purification.

Lipocalins are characterized by highly conserved 3D structures forming an internal pocket with the ability to bind a range of small hydrophobic molecules. The fluorescence spectra of native and denatured $\alpha_1\text{m}$ indicate that the chromophores are buried or semi-buried in the hydrophobic core of the protein. According to a model based on the sequence of $\alpha_1\text{m}$ in comparison with the lipocalin ERABP, the three chromophore-carrying Lys residues are closely spaced and semiburied near the entrance of the predicted lipocalin pocket. This suggests that the 282 amu substance is also located in the pocket or near its entrance. Thus, the substance could be the hitherto unknown lipocalin ligand of $\alpha_1\text{m}$. A possible explanation for its presence in the lipocalin pocket is that it is subjected to transport by $\alpha_1\text{m}$. Another possibility is that the 282 amu substance participates in the formation or turnover of the chromophores, either as a substrate, an intermediate, or a degradation product. Such a reaction could very well be mediated by the two functional groups, hydroxyl and amine, for example by formation of Schiff bases.

Other investigators have shown evidence that a chromophore is bound to Cys34 (Escribano et al., 1991). In this study, no colored peptides containing Cys34 were found. The purification protocol used in this study may be insufficient for purification of colored peptides containing Cys34. Alternatively, the chromophores bound to Cys34 may be of an even more heterogeneous nature than the chromophores linked to Lys118, Lys130, and Lys92, thus escaping detection. The peptide $\text{W}_{25}\text{YNLAIGSTCPWLK}$ was identified by its expected molecular mass, suggesting that at least part of the Cys34 is unmodified in the native protein. Future studies will hopefully clarify the relationship between the chromophores bound to Lys118, Lys130, Lys92, and Cys34.

A recent report describes the finding of unusually high levels of glycation in $\alpha_1\text{m}$ (Bonay et al., 1997). Glycation is a slow, non-enzymatic reaction between sugars and primary amino groups in proteins, i.e., ϵ -amino groups of Lys residues, leading to formation of brown-colored advanced glycation end products (AGE) (Thorpe & Baynes, 1996; Chappey et al., 1997; Deyl & Miksik, 1997). The properties of the $\alpha_1\text{m}$ chromophores described here resemble those described for AGEs, but there are several differences which suggest that they are not glycation products: (1) $\alpha_1\text{m}$ is by far more colored than glycated proteins, suggesting a much more rapid formation of the chromophore of $\alpha_1\text{m}$. Indeed, insect cells infected with a virus encoding $\alpha_1\text{m}$ express a yellow-brown $\alpha_1\text{m}$ immediately after infection (Åkerström et al., 1995; Wester et al., 1997) and intracellular $\alpha_1\text{m}$ purified from place liver is yellow-brown (Lindqvist & Åkerström, 1999). (2) Incubation of $\alpha_1\text{m}$ with sugars in vitro gave no evidence of a higher rate of glycation of $\alpha_1\text{m}$ than of other plasma proteins. (3) No *cis*-diol hydroxyl groups, i.e., early products of glycation, were found in the protein. Therefore,

the $\alpha_1\text{m}$ chromophores may be similar to late glycation products, AGEs, but available data indicate that they are formed and attached to the $\alpha_1\text{m}$ polypeptide by a different, but perhaps related mechanism.

$\alpha_1\text{m}$ has a strong complex-forming ability. In man and in other species, approximately 50% of the total $\alpha_1\text{m}$ is found as covalent complexes with several different plasma proteins. The results in this study show that the $\alpha_1\text{m}$ subpopulation linked to IgA, the dominating complex-partner in human plasma, was almost completely devoid of coloration. Similarly, the quantitatively dominating complex in rat plasma, α_1 -inhibitor-3/ $\alpha_1\text{m}$, seems to be uncolored (Falkenberg et al., 1990). IgA was shown to be linked via Cys34 to $\alpha_1\text{m}$ (Calero et al., 1994). Based on the above-mentioned 3D model, Cys34 is located in a large omega loop that forms a lid, which may "close" the internal cavity of lipocalins (Flower, 1995, 1996). It can thus be speculated that complex formation between $\alpha_1\text{m}$ and other plasma proteins alters the shape of the lid, thereby affecting the formation or turnover of the chromophores as well as the association of the 282 amu compound.

Materials and methods

Proteins and reagents

Human plasma was obtained from healthy blood donors. Monoclonal mouse anti- $\alpha_1\text{m}$, BN 11.10, which binds to rat and human $\alpha_1\text{m}$, was prepared and purified as described (Babiker-Mohamed et al., 1991). Modified porcine trypsin and pepsin were from Promega (Madison, Wisconsin). HPLC grade acetonitrile and water were obtained from Baker (Burdick and Jackson Division, Muskegon, Michigan). Trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, Illinois). Formic acid was purchased from Fisher Scientific (Fair Lawn, New Jersey). Radiolabeled carbohydrates were from NEN (Boston, Massachusetts) research products or from Boehringer Mannheim (Mannheim, Germany). $\alpha_1\text{m}$ was purified from the urine of patients with tubular proteinuria as described earlier (Åkerström et al., 1995).

Peptide purification

Lyophilized $\alpha_1\text{m}$ (1–10 mg) was dissolved in 7 mL of a buffer (100 mM NaCl, 50 mM Tris-HCl, pH 8.0) containing 6 M guanidine and 5 mM dithioerythritol, and incubated for 1 h at 23 °C in darkness. After this, iodoacetamide was added to 10 mM, and the solution incubated for another 30 min at 23 °C in darkness. The sample was dialyzed exhaustively against 20 mM ammonium bicarbonate, pH 7.4, and concentrated to ~1 mL by vacuum centrifugation. The reduced and S-carboxymethylated $\alpha_1\text{m}$ was digested with modified porcine trypsin for 3 h at 37 °C using an enzyme/substrate ratio of 1:80, freeze-dried and dissolved in 1 mL 0.1% TFA. Colored peptides were purified by repeated RP-HPLC on a Pharmacia (Uppsala, Sweden) Smart system equipped with a Brownlee (Aquapore RP-300, 220 × 2.1 mm), Brownlee (Vydac C18 200 × 2.1 mm), Beckman (Ultrasphere 250 × 2.0 mm), or Phenomenex (Torrence, California) (Nucleosil C18 250 × 2.0 mm) column, eluting with a linear gradient of 0.1% TFA to 90% acetonitrile/0.07% TFA at a flow rate of 200 $\mu\text{L}/\text{min}$. Some fractions were further purified by a Brownlee (Vydac C18 200 × 2.1 mm) column followed by a Phenomenex (Nucleosil C18 250 × 2.0 mm) column eluting with a linear gradient of 0.1% TFA to 75%

2-propanol/ 0.07% TFA at a flow rate of 125 μ L/min. Fractions were collected manually based on a 350 nm analogue signal from the μ peak detector (Pharmacia). The purity of the colored peptides was determined by Edman degradation. The colored peptides were pure when only one homogeneous, sharp peak of co-migrating absorbance at both 350 and 220 nm were seen and the ratio of 220/350 nm absorbance was lower than 2. The absorbances at 220 and 350 nm were digitized and recorded on a computer for post-run analysis. In some experiments, the reduced, carboxymethylated, and trypsin-digested α_1 m was subjected to size exclusion chromatography on a Pharmacia Superdex HR 10/30 column eluting with 0.1% TFA in H₂O at a flow rate of 25 μ L/min. Fractions were collected according to the absorbance at 350 nm and colored peptides were further purified by RP-HPLC as described above.

Amino acid sequence analysis

Automated Edman degradation was carried out in an Applied Biosystems 477 A sequencer with on-line detection of PTH amino acids using an Applied Biosystems 120A HPLC.

Electrospray ionization mass spectrometry (ES-MS) analysis of chromophore peptides

The analysis was performed using a VG Biotech BIO-Q instrument with quadrupole analyzer. Sample aliquots of 50 μ L were injected into the instrument source. Elution was carried out using a mixture of 50% aqueous acetonitrile containing 0.1% TFA, at a flow rate of 10 μ L/min. Calibration was performed with cesium iodide. Alternatively, analysis was performed using an Esquire-LC ion-trap mass spectrometer (Bruker, Bremen, Germany) with a Bruker nano spray interface, in positive ionization mode. The samples were placed in a gold plated glass capillary needle and inserted into the nano spray interface. The solvent was a mixture of 50% aqueous methanol (Merck, Darmstadt, Germany) containing 0.1% trichloroacetic acid (TCA).

Mass spectrometry peptide mapping

Lyophilized α_1 m (~50 μ g) was dissolved in 50 μ L of 0.2 M ammonium bicarbonate, pH 8.0 and digested with trypsin using an enzyme/substrate ratio of 1:10, for 4 h at 37 °C. The pH was then adjusted to 2 by the addition of TFA. An aliquot of the digest (5 μ L) was subjected to RP-HPLC (Deltabond ODS 150 $\times$ 1 mm, Keystone Scientific, Bellefonte, Pennsylvania) using an Isco (Lincoln, Nebraska) microbore HPLC system. The digest was eluted at 30 μ L/min using 0.025% TFA and 90% acetonitrile containing 0.025% TFA. The effluent was split evenly into two streams. One stream was monitored at 350 nm and the other was delivered directly to the source of the mass spectrometer. Mass spectra were recorded on a Fisons Quattro-BQ triple quadrupole mass spectrometer equipped with a pneumatically assisted electrostatic ion source operating at atmospheric pressure. The data were collected in the continuum mode from mass/charge (m/z) ratio 230–1,450 with a scan time of 5 s. Spectra were calibrated using polyethylene glycol.

Glycation analysis

One hundred twenty to 600 μ g of α_1 m, human serum albumin, or orosomucoid were glycated in vitro in 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) containing either 0.2 nM [³H]-glucose, 0.2 nM [³H]-galactose, 0.2 nM [¹⁴C]-ribose, or 0.2 nM [¹⁴C]-fructose at 37 °C for various amounts of time. The glycated protein was then separated from free sugar on a PD-10 column (Pharmacia) eluted with PBS. Fifty microliter fractions were collected and the radioactivity measured in a β -counter. Alternatively, 200 μ g of α_1 m, human serum albumin, or orosomucoid were incubated at 37 °C with 0.5 M glucose or 0.5 M fructose in PBS. The absorbance spectra of the proteins were measured at various time points in a Uvicon 930 Spectrophotometer (Kontron Instruments, Neufahrn, Germany). Human serum albumin was glycated by incubation with 250 mM glucose at 37 °C in PBS for 30 days and then separated from free sugar on a PD-10 column. Human urinary α_1 m (200 μ g) or glycated or nonglycated human serum albumin (200 μ g) was applied to a 1 mL Glyco-gel II Boronate Affinity Gel column (Pierce, Rockford, Illinois), and bound proteins eluted according to the instructions supplied by the manufacturer. The amounts of protein in the bound and nonbound fractions were quantified by radioimmunoassay, absorbance, and SDS-PAGE.

Construction of a 3D model of α_1 m

Coordinates for the rat ERABP were retrieved as a Brookhaven Database Protein Data Bank (PDB) file, accession code 1EPA. The file was used together with the amino acid sequence of bovine α_1 m (Lindqvist & Åkerström, 1996) to generate a 3D model of α_1 m, utilizing the Swiss-PdbViewer version 3.01 program linked to Swiss-Model, an automated homology modeling server (Guex & Peitsch, 1997).

Fluorescence spectra

Fluorescence spectra were obtained with a Perkin Elmer LB50 Fluorescence spectrometer. Excitation was made at 360 nm for the unknown chromophore. The excitation slit width was 5 nm, the emission slit width was 3 nm, and the scan speed was 150 nm/min. Nonreduced and nondenatured α_1 m was measured at 36.4 μ M in 50 mM Mes (2-morpholino ethane sulfonic acid/sodium salt) buffer, pH 6.3. The reduced and denatured samples were 36.4 μ M with regard to α_1 m, 2.6 mM with regard to Mesna (2-mercapto-ethane-sulfonic acid/sodium salt) and 9 M with regard to urea (ultra pure, Gibco BRL, Gaithersburg, Maryland/Life Technologies, Rockville, Missouri) in 50 mM Mes-buffer, pH 6.3. The samples were incubated 1 h at 37 °C prior to analysis. All chemicals were from Sigma (St. Louis, Missouri). Controls were measured in 50 mM Mes buffer with or without 9 M urea and 2.6 mM Mesna.

Purification of IgA/ α_1 m and preparation of the α_1 m component of the complex

Immunosorbent affinity chromatography of human plasma with monoclonal mouse anti- α_1 m, BN11.10, immobilized to Affigel Hz (20 mg/mL) followed by Sephacryl S-300 size exclusion chromatography, was done as described (Berggård et al., 1997). Fractions containing IgA/ α_1 m, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, see below), were concentrated and dialyzed. One-half milliliter of IgA/ α_1 m (3 mg/mL) in 50 mM sodium-acetate, pH 4.5, was partly digested with pepsin for 20 min at 20 °C using an enzyme/substrate ratio of 1:50. The reaction was stopped by addition of 2 M Tris-HCl buffer as

described earlier (Calero et al., 1994). The mixture was separated by Sephacryl S-200 size exclusion chromatography. The two protein fractions were analyzed by SDS-PAGE as described (Laemmli, 1970). Following electrophoresis, the proteins were either stained with Coomassie Brilliant Blue, or transferred to a polyvinylidene difluoride membrane ("Immobilon," Millipore) as described (Matsudaira, 1987) and subjected to Edman degradation.

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References

- Åkerström B, Bratt T, Enghild JJ. 1995. Formation of the α_1 -microglobulin chromophore in mammalian and insect cells: A novel post-translational mechanism? *FEBS Lett* 362:50–54.
- Åkerström B, Lögdberg L. 1990. An intriguing member of the lipocalin protein family: α_1 -microglobulin. *Trends Biochem Sci* 15:240–243.
- Babiker-Mohamed H, Forsberg M, Olsson ML, Winquist O, Nilson BH, Lögdberg L, Åkerström B. 1991. Characterization of monoclonal anti- α_1 -microglobulin antibodies: Binding strength, binding sites, and inhibition of lymphocyte stimulation. *Scand J Immunol* 34:655–666.
- Berggård T, Thelin N, Falkenberg C, Enghild JJ, Åkerström B. 1997. Prothrombin, albumin and immunoglobulin A form covalent complexes with α_1 -microglobulin in human plasma. *Eur J Biochem* 245:676–683.
- Bonay P, Solis J, de la Calle H, Fresno M, Grubb A, Mendez E. 1997. Massive glycation of protein HC, a low molecular weight lipocalin, in non-diabetic individuals. *FEBS Lett* 416:276–280.
- Bratt T, Olsson H, Sjöberg EM, Jergil B, Åkerström B. 1993. Cleavage of the α_1 -microglobulin-bikunin precursor is localized to the Golgi apparatus of rat liver cells. *Biochim Biophys Acta* 1157:147–154.
- Calero M, Escribano J, Grubb A, Mendez E. 1994. Location of a novel type of interpolypeptide chain linkage in the human protein HC-IgA complex (HC-IgA) and identification of a heterogeneous chromophore associated with the complex. *J Biol Chem* 269:384–389.
- Chapppay O, Dosquet C, Wautier MP, Wautier JL. 1997. Advanced glycation end products, oxidant stress and vascular lesions. *Eur J Clin Invest* 27:97–108.
- Deyl Z, Miksik I. 1997. Post-translational non-enzymatic modification of proteins. I. Chromatography of marker adducts with special emphasis to glycation reactions. *J Chromatogr B Biomed Sci Appl* 699:287–309.
- Ekström B, Berggård I. 1977. Human α_1 -microglobulin. Purification procedure, chemical and physicochemical properties. *J Biol Chem* 252:8048–8057.
- Ekström B, Peterson PA, Berggård I. 1975. A urinary and plasma α_1 -glycoprotein of low molecular weight: Isolation and some properties. *Biochem Biophys Res Commun* 65:1427–1433.
- Enghild JJ, Thøgersen IB, Pizzo SV, Salvesen G. 1989. Analysis of inter- α -trypsin inhibitor and a novel trypsin inhibitor, pre- α -trypsin inhibitor, from human plasma. Polypeptide chain stoichiometry and assembly by glycan. *J Biol Chem* 264:15975–15981.
- Escribano J, Grubb A, Calero M, Mendez E. 1991. The protein HC chromophore is linked to the cysteine residue at position 34 of the polypeptide chain by a reduction-resistant bond and causes the charge heterogeneity of protein HC. *J Biol Chem* 266:15758–15763.
- Escribano J, Lopez-Otin C, Hjerpe A, Grubb A, Mendez E. 1990. Location and characterization of the three carbohydrate prosthetic groups of human protein HC. *FEBS Lett* 266:167–170.
- Falkenberg C, Enghild JJ, Thøgersen IB, Salvesen G, Åkerström B. 1994. Isolation and characterization of fibronectin- α_1 -microglobulin complex in rat plasma. *Biochem J* 301:745–751.
- Falkenberg C, Grubb A, Åkerström B. 1990. Isolation of rat serum α_1 -microglobulin. Identification of a complex with α_1 -inhibitor₃, a rat α_2 -macroglobulin homologue. *J Biol Chem* 265:16150–16157.
- Flower DR. 1995. Multiple molecular recognition properties of the lipocalin protein family. *J Mol Recognit* 8:185–195.
- Flower DR. 1996. The lipocalin protein family: structure and function. *Biochem J* 318:1–14.
- Grubb AO, Lopez C, Tejler L, Mendez E. 1983. Isolation of human complex-forming glycoprotein, heterogeneous in charge (protein HC), and its IgA complex from plasma. Physicochemical and immunochemical properties, normal plasma concentration. *J Biol Chem* 258:14698–14707.
- Guex N, Peitsch MC. 1997. SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. *Electrophoresis* 18:2714–2723.
- Hanley S, McInerney JO, Powell R. 1995. Sequence analysis and evolutionary aspects of piscine α_1 -microglobulin/bikunin mRNA transcripts. *Mol Mar Biol Biotechnol* 4:105–109.
- Hanley S, Powell R. 1994. Sequence of a cDNA clone encoding the Atlantic salmon α_1 -microglobulin/bikunin protein. *Gene* 147:297–298.
- Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Leaver MJ, Wright J, George SG. 1994. Conservation of the tandem arrangement of α_1 -microglobulin/bikunin mRNA: Cloning of a cDNA from plaice (*Pleuronectes platessa*). *Comp Biochem Physiol Biochem Mol Biol* 108:275–281.
- Lindqvist A, Åkerström B. 1996. Bovine α_1 -microglobulin/bikunin. Isolation and characterization of liver cDNA and urinary α_1 -microglobulin. *Biochim Biophys Acta* 1306:98–106.
- Lindqvist A, Åkerström B. 1999. Isolation of plaice (*Pleuronectes platessa*) α_1 -microglobulin: Conservation of structure and chromophore. *Biochim Biophys Acta* 1430:222–233.
- Matsudaira P. 1987. Sequence from picomole quantities of proteins electrophoretically onto polyvinylidene difluoride membranes. *J Biol Chem* 262:10035–10038.
- Salier JP. 1990. Inter- α -trypsin inhibitor: Emergence of a family within the Kunitz-type protease inhibitor superfamily. *Trends Biochem Sci* 15:435–439.
- Sansom CE, North AC, Sawyer L. 1994. Structural analysis and classification of lipocalins and related proteins using a profile-search method. *Biochim Biophys Acta* 1208:247–255.
- Tejler L, Grubb AO. 1976. A complex-forming glycoprotein heterogeneous in charge and present in human plasma, urine, and cerebrospinal fluid. *Biochim Biophys Acta* 439:82–94.
- Thøgersen IB, Enghild JJ. 1995. Biosynthesis of bikunin proteins in the human carcinoma cell line HepG2 and in primary human hepatocytes. Polypeptide assembly by glycosaminoglycan. *J Biol Chem* 270:18700–18709.
- Thorpe SR, Baynes JW. 1996. Role of the Maillard reaction in diabetes mellitus and diseases of aging. *Drugs Aging* 9:69–77.
- Wester L, Johansson MU, Åkerström B. 1997. Physicochemical and biochemical characterization of human α_1 -microglobulin expressed in baculovirus-infected insect cells. *Protein Expr Purif* 11:95–103.