Human α-1-Microglobulin Is Covalently Bound to Kynurenine-derived Chromophores*

Received for publication, July 21, 2004, and in revised form, September 14, 2004
Published, JBC Papers in Press, September 27, 2004, DOI 10.1074/jbc.M408242200

Alberto Sala‡, Monica Campagnoli‡, Eleonora Perani§, Assunta Romano‡, Sara Labò‡, Enrico Monzani‡, Lorenzo Minchiotti‡, and Monica Galliano‡

From the ‡Department of Biochemistry, University of Pavia, Viale Taramelli 3B, the §Centro Grandi Strumenti, University of Pavia, Via Bassi 21, and the ¶Department of General Chemistry, University of Pavia, Viale Taramelli 12, 27100 Pavia, Italy

α-1-Microglobulin carries a set of covalently linked chromophores that give it a peculiar yellow-brown color, fluorescence properties, and both charge and size heterogeneity. In this report it is shown that these features are due to the adducts with the tryptophan metabolite, 3-hydroxykynurenine, and its autoxidation products and that the modification is more pronounced in the protein isolated from urine of hemodialysed patients. The light yellow amino acid fluid α-1-microglobulin acquires the optical properties and charge heterogeneity of the urinary counterpart following incubation with kynurenines. The colored amino acid adducts of urinary and amniotic fluid α-1-microglobulins were separated by chromatography after acid hydrolysis and analyzed by mass spectrometry. Human serum albumin samples, native and treated with 3-hydroxykynurenine in the presence of oxygen, were used as a control. The retention times and mass fragmentation products were compared, and a lysyl adduct with hydroxantomathin was identified in the urinary α-1-microglobulin and in the modified albumin samples. The more extensive modification of the urinary protein appears to be correlated with uremia, a condition in which the catabolism of tryptophan via the kynurenine pathway is increased, and the consequent rise in the concentration of its derivatives is accompanied by the oxidative processes due to the hemodialysis treatment. The oxidative derivatives of 3-hydroxykynurenine, which are known to act as protein cross-linking agents, are the likely cause of the propensity of urinary α-1-microglobulin to form dimers and oligomers. This process, as well as the redox properties of these metabolites, may contribute to the toxic effects of the kynurenine species.

α-1-Microglobulin (α-1-m),1 also known as protein HC, is a widely distributed glycosylated protein that on the basis of sequence homology has been included in the lipocalin family. This large group of predominantly extracellular molecules shares a common β-barrel fold but has been assigned different functional roles (1). α-1-m is present in many tissues and, although its function is not known, several reports hint at a role as an immunomodulator (2, 3). Its single polypeptide chain consists of 183 residues (4) and contains one O-linked and two N-linked oligosaccharide moieties (5). The synthesis takes place mainly in the liver and, in humans, the α-1-m gene has been mapped to chromosome 9 in a region where other lipocalin genes are clustered (4, 6). As for all other species, it encodes also bikunin, the light chain of a plasma proteinase inhibitor family (7), and the precursor polypeptide chain contains an internal basic tripeptide recognized by a specific endopeptidase. The cleavage occurs in the Golgi apparatus before secretion and, so far, no functional correlation between the lipocalin and bikunin has been found. α-1-m is then catabolized in the proximal tubule cells of the kidneys, and its concentration increases in urines of patients with renal failure (8). The protein has thus been recognized as a very sensitive marker for proximal tubular dysfunction (9). Due to its relative abundance in the urines of hemodialysed patients, the majority of the structural and biochemical studies (reviewed in Ref. 10) have been carried out using the protein isolated from this source. The molecule has unique features that, so far, remain unexplained: it has a strong tendency to bind to other proteins, which results in the formation of complexes that are too large to allow removal from the circulation via glomerular filtration. Furthermore, it carries a set of covalently linked chromophores that give it a peculiar yellow-brown color, fluorescence properties, and both charge and size heterogeneity. Although the nature of the bound compound has not been established, the single free cysteine (11) and three lysines (12) have been identified as the modified residues.

In a previous study we isolated α-1-microglobulin from amniotic fluid (5) and observed that its light yellow color was not more intense than that of the other proteins from the same source, which suggested the presence of a lower degree of chromophore modification with respect to the urinary protein. We therefore decided to examine the possibility that the intense coloration, and related properties, of the urinary protein might be associated with uremia, a condition in which a great number of endogenous metabolites that are ordinarily excreted in urine accumulate in blood. Among these are the products of tryptophan metabolism that occur via the kynurenine pathway. Several recent reports have shown that the degradation of tryptophan is altered in patients with chronic renal failure and that there is an increased level of kynurenine (Kyn) that is further converted into a series of compounds with neurotoxic properties (13). Kyn and its derivatives are also present in the lens (14) where, following their reaction with the crystallins,
produce covalent brown-colored adducts with broad long wave-
length-absorbing characteristics and with the development of
non-tryptophan fluorescence (15). The modification involves
histidine, lysine, and, to a lesser extent, cysteine residues and
appears to play a role in cataract formation. In addition, the
binding of 3-hydroxykynurenine (3-OHkyn) oxidation products
is accompanied by aggregation and the formation of higher
molecular weight species (16).

In the present work we show that 3-OHkyn and its autoxi-
dation products form covalent adducts with human α-1-m and
that the modification, affecting more extensively the protein
isolated from the urine of patients with renal failure, accounts
for the peculiar optical properties as well as for the charge
and size heterogeneity of the lipocalin.

EXPERIMENTAL PROCEDURES

Materials and Methods—Amino acid derivatives (N-acetyl-L-histi-
dine, N-acetyl-L-lysine, and N-acetyl-L-cysteine) were obtained
from Calbiochem-Novabiochem AG. 3-Hydroxykynurenine and L-kynurenine
sulfate were from Sigma. All organic solvents and acids were HPLC
grade, and Milli Q water was used in the preparation of HPLC solu-
tions. All other chemicals were of analytical grade.

Protein Purification—α-1-Microglobulin was isolated from the urine
of hemodialyzed patients as previously described (5). Human
amniotic fluid was obtained from discarded amniocentesis samples
(2 liters) of hemodialyzed patients as previously described (5). Human
eluting after the albumin peak were pooled, equilibrated in 6.25 mM
ammonium acetate, pH 6.5 (solvent A), and resol-
ved with a linear gradient 0–50% of solvent B (80% acetonitrile/H2O,
4 mM ammonium acetate) which individual peaks were collected and lyoph-
ilized. Aliquots were then dissolved in 0.1% trifluoroacetic acid
and submitted to liquid chromatography mass spectrometry (LC/MS). Elec-
tronspray ionization tandem mass spectrometry (ESI-MS/MS) of ly-
sylkynurenine (Lys-Kyn), at m/z 338 [M+H]+, yielded ion fragments at
m/z 209, 203, 192, 147, and 128. ESI-MS/MS of histidylkynurenine
(His-Kyn), at m/z 347 [M+H]+, yielded ion fragments at m/z 192, 174,
and 156. Using the same protocol the protected amino acids were
reacted with 3-OHkyne and, the adducts were separated by RP-HPLC
and submitted to ESI/MS/MS.

Acid Hydrolysis—The proteins (3 mg) were hydrolyzed with 6 M HCl
for 24 h at 110 °C in evacuated hydrolysate tube and lyophilized. Sam-
ple were then dissolved in 400 μl of 4 mM ammonium acetate, pH 6.5,
alcohols were added to 6.5 with 0.5 M NaOH, resolved by
RP-HPLC on a Vydac C18 column as described for the standard amino
acid adducts. The elution was monitored at 360 nm, and the colored
peaks were manually collected and lyophilized. For LC-ES/MS analyses,
lyophilized samples were dissolved in 0.1% trifluoroacetic acid.

Liquid Chromatography/Electrospray Ionization Mass Spectrome-
try—Mass spectra were obtained using an LQD DECA ion trap mass
spectrometer equipped with an electrospray ionization (ESI) source
and controlled by Xcalibur software 1.3 (Thermo-Finnigan, San Jose, CA).
Experiments were carried out in positive ion mode under constant
instrumental conditions: source voltage 4.5 kV, capillary voltage
diameter 20 V, capillary temperature 200 °C, tube lens voltage −5 V. The system
was run in automated LC-MS mode, and the HPLC used was a Surveyor LC
system (Thermo-Finnigan) equipped with a Waters Symmetry300C18
(3.5 mm, 2.1 × 100 mm column. Samples (20 μl) were applied at an
injection speed of 8 μl/min and resolved with a gradient of 0–50% linear
100-min gradient using 0.1% trifluoroacetic acid in water as solvent A
and 0.1% trifluoroacetic acid in acetonitrile as solvent B. External
 calibration of the mass scale was achieved by injection of caffeine (m/z
195), MRF tetrapeptide (m/z 524), and Ultramark (m/z 1022, 1122,
1222, 1322, 1422, 1522, 1622, 1722, and 1822). Mass spectra were
acquired in the m/z range of 300–1000 atomic mass units by scanning
the magnetic field in 200 ms. MS/MS spectra were obtained by collision-
induced dissociation; studies in the ion trap were performed with an
isolation width of 3 Thomson (m/z), the activation amplitude
was around 35% of ejection radio frequency amplitude that corresponds to
1.58 V.

RESULTS

Electrophoretic and Optical Properties of Amniotic Fluid and
Uriney α-1-m—α-1-Microglobulin was isolated from urines of
hemodialyzed patients and from amniotic fluid and was found to
be homogeneous on the basis of the N-terminal sequence
determination and SDS-PAGE analysis, which showed the
presence of a 30-kDa band (Fig. 1). However, after prolonged
storage, the urinary protein showed a marked propensity to
form SDS-resistant dimers and higher order oligomers while
the amniotic fluid form remained unchanged (data not shown).

![Fig. 1. SDS-PAGE of purified α-1-microglobulin.](image-url)
Dimers of the urinary protein (Fig. 1, lane 2) become evident almost immediately after the last gel filtration purification step in Tris-HCl buffer 20 mM, pH 8.0, whereas oligomer formation was observed only after several months of storage in the same buffer (Fig. 1, lane 3). The isoelectric focusing patterns (Fig. 2) display several discrete bands in the range between pH 4.0 and 5.4 in agreement with the well known charge heterogeneity of the protein that, in earlier reports, was named “HC,” the acronym meaning “heterogeneous in charge.” However, the smear of the sample toward the anode, which was yellow-brown in the gel before staining, could only be noticed in the urinary protein pattern (Fig. 2, lane 1). We have previously reported that the presence of a highly heterogeneous mixture of differently branched glycans and the existence of three different polypeptide chains, due to varying degrees of proteolytic cleavage at the C terminus, contribute to the extensive charge heterogeneity of these proteins (5). However, the bands focusing in the anodic side of the gel, which were almost absent in the amniotic fluid form (Fig. 2, lane 2), appeared to be correlated with the degree of coloration of the urinary protein. The UV-visible spectrum of the yellow-brown protein isolated from urine displays the characteristic broad absorbance band in the visible region between 300 and 600 nm. When analyzed in detail, the spectrum of a concentrated solution (10 mg/ml) of the protein shows the presence of shoulders, approximately at 350, 420, and 490 nm (Fig. 3A). The fluorescence spectrum of urinary α-1-m exhibited a maximum emission intensity at 455 nm (Fig. 4, lane 2), whereas the amniotic fluid counterpart had a lower absorbance at 360 nm, almost none above 450 nm (Fig. 3B, lane 1), and an emission spectrum with the same shape but a lower intensity (Fig. 4, lane 1). The relative amount of bound chromophore was estimated by measuring the ratio between the absorbance at 360 and 280 nm. The value of 0.48 was obtained for the urinary protein and of 0.21 for the amniotic fluid counterpart. These results indicate that, although the optical and electrophoretic properties of the two proteins share common features, the lipocalin from the urine of hemodialyzed patients contains greater quantities and more heterogeneous amino acid side chain of Kyn yields an unsaturated ketone that is susceptible to attack by nucleophilic amino acids, such as His, Lys, and Cys and that the process is favored when the pH is increased to 9.5 (25). The amniotic fluid α-1-m was treated with Kyn in carbonate buffer at pH 9.5 (15) and, after gel filtration at pH 7.0, the modification was verified following the optical properties of the adduct. The UV-visible spectrum of α-1-m shows that, following incubation, a more pronounced absorption maximum appears at 360 nm (Fig. 3B, lane 2) and that the fluorescence intensity is increased, with a maximum emission at 457 nm (Fig. 4, lane 3).

The reaction with Kyn was carried out, under the same
derivatives.

pathway (26), explains the presence of the ion at

bound to a Lys residue, according to its major decomposition

Elimination of the imine side chain of deaminated kynurenine

adducts showed the presence of the expected ion fragments.

His-Kyn (15), confirms their identification as the Lys-Kyn (15),

the extracted ion currents for the protonated molecular ions

correlation between the absorbance at 360 nm (Fig. 7A)

shown). These results show that amniotic α-1-microglobulin,

as well as HSA, reacts with Kyn. The reaction promoted an

enhancement of the fluorescence emitted by the amniotic fluid protein but the band position remained invariant and very close to that of the protein isolated from the urine of hemodialyzed patients. The UV-visible spectrum of the latter, which is considerably more complex, compared with that of the modified amniotic fluid protein, indicates the presence of a set of Kyn-derived chromophores.

It has been shown that kynurenic adducts with His, Lys, and, to a lesser extent, with Cys residues are stable under the conditions used for total hydrolysis of proteins and can be analyzed by RP-HPLC following the absorbance at 360 nm (15). Aliquots (3 mg) of amniotic α-1-m and of HSA were subjected to acid hydrolysis prior and after incubation with Kyn (M, 208), and the hydrolysates were analyzed by RP-HPLC and LC-ESI/MS. Reference standard adducts of His, Lys, and Cys were prepared according to Vazquez et al. (15) and examined by RP-HPLC and LC-ESI/MS to facilitate their identification in the protein samples. The HPLC traces showed the absence of any colored peak in the hydrolysate of native HSA (Fig. 6A, line a), whereas some minor peaks absorbing at 360 nm were present in the hydrolysate of the native amniotic α-1-m (Fig. 6C).

After treatment with Kyn a major colored peak, corresponding to Lys-Kyn derivative, and a second, less intense, eluting as His-Kyn derivative, were found in both samples (data not shown). The hydrolysates were then examined by LC-MS and correlation between the absorbance at 360 nm (Fig. 7A), and the extracted ion currents for the protonated molecular ions confirmed their identification as the Lys-Kyn (m/z 338) and the His-Kyn (m/z 347) derivatives. The ESI-MS/MS spectra of the adducts showed the presence of the expected ion fragments. Elimination of the imine side chain of deaminated kynurenine bound to a Lys residue, according to its major decomposition pathway (26), explains the presence of the ion at m/z 203 (Fig. 7B) and confirms the site of the covalent attachment (15). These results establish that, following reaction with Kyn, the Lys and His residues of amniotic α-1-m and of HSA form colored adducts that can be identified after acid hydrolysis by HPLC monitoring of the absorbance at 360 nm. No such absorbance is present in the hydrolysate of native human serum albumin that can thus be used as a reference to monitor the modification. The relative amount of the modified amino acids in the hydrolysates reflects the residue abundance in the proteins. The low stability of the adduct and the presence of only one free Cys in the sequence of both α-1-m and albumin account for the undetectability of such adduct.

LC/MS Analysis of Urinary and Amniotic Fluid α-1-m after Acid Hydrolysis—The HPLC trace of the acid hydrolysate obtained from the urinary lipocalin (Fig. 6B) showed the presence of several peaks absorbing at 360 nm, but none of them could be clearly identified as a Kyn adduct on the basis of their retention times or by ESI/MS analysis. The ESI/MS spectra of the urinary and amniotic fluid hydrolysates were then compared, and a charged species at m/z 389 [M+H]⁺, eluting under peaks 2’ and 1’ respectively, was found in both samples (Fig. 8). They were then submitted to ESI-MS/MS, and Fig. 9 shows the fragmentation pattern of the singly charged ion at m/z 389 [M+H]⁺ from the amniotic fluid.
protein that was almost identical to that of its urinary counterpart (data not shown). The product ion of largest mass, at \( m/z \) 343, derives from the molecular ion after the loss of CO2 \([\text{M}/\text{H}]^{-1000} - \text{H-CO2}\] /\( \text{H}11001 \). The product ions at \( m/z \) 208, 191, 162, and 136 were identified as derived from 3-OHKyn. The sequential loss of ammonia accounts for the ions at \( m/z \) 208 \([3\text{-OHKyn}/\text{H}]^{-1100} - \text{H-NH3}\] /\( \text{H}11001 \) and at \( m/z \) 191 \([3\text{-OHKyn}/\text{H}]^{-1100} - 2\text{H-NH3}\] /\( \text{H}11001 \), the elimination of H2O and CO from the deaminated ion \([3\text{-OHKyn}/\text{H}]^{-1100} - \text{H-NH3-H2O-CO}\] /\( \text{H}11001 \) produces the peak at \( m/z \) 162, and the cleavage occurring at the carbonyl group generates the species at \( m/z \) 136 \([3\text{-OHKyn} + \text{H-CH2CH(NH2)COOH}]^{-} \). This fragmentation pattern is in agreement with the known decomposition pathway of the Kyn molecular ion (27). The fragment ion at \( m/z \) 147 \([\text{Lys} + \text{H}]^{-} \) indicated that the adduct incorporated a lysyl residue. These results show that the \( \alpha\)-1-m covalently bound adducts are stable to acid hydrolysis and can be detected in the HPLC trace following their absorbance at 360 nm. The same colored adduct was found in both samples; it is

![Image](https://example.com/image.png)

**FIG. 7.** LC/MS analysis of amniotic fluid \( \alpha\)-1-microglobulin incubated with Kyn and submitted to acid hydrolysis. Panel A shows the UV absorbance at 360 nm of the total acid hydrolysate dissolved in 0.1% trifluoroacetic acid (solvent A) and resolved by means of a 0–50% linear 100 min gradient using 0.1% trifluoroacetic acid in acetonitrile as solvent B. The colored peaks at \( R_t = 7.05 \) min and \( R_t = 8.01 \) min were identified as Lys-Kyn \((m/z 338)\) and His-Kyn \((m/z 347)\), respectively. Panel B shows the MS/MS spectrum of \( m/z \) 338 \([\text{M} + \text{H}]^{-} \) corresponding to the protonated molecular ion at \( R_t = 7.05 \). Product ions are characteristic of the Lys-Kyn adduct.

![Image](https://example.com/image.png)

**FIG. 8.** LC/MS analysis of peaks 2, 2', and 1' obtained after RP-HPLC separation of the acid-hydrolyzed proteins. Samples were dissolved in 0.1% trifluoroacetic acid and analyzed by LC-ESI/MS. Panels A–C show the chromatograms of the extracted ion current for \( m/z \) 389 \([\text{M} + \text{H}]^{-} \) identified in: A, peak 1' from amniotic fluid \( \alpha\)-1-m; B, peak 2' from urinary \( \alpha\)-1-m; C, peak 2 from treated HSA.
Therefore, amniotic fluid attributed to the formation of autoxidation derivatives which the formation of tanned products (29). The coloration has been proteins and that this covalent attachment is associated with (28). It has also been shown that 3-OHKyn reacts with lens nol, which is its most abundant metabolite in biological fluids ical conditions Kyn is converted into 3-OHKyn, an aliphatic side chain to yield the oxidative products (16). Deamination of the 3-OHKyn in the presence of oxygen to allow the formation of 3-OHKyn in the presence of oxygen the light yellow am-

nary lipocalin in the 350- to 500-nm range, thus suggesting that the heterogeneous modification of the latter is due to the oxidation products of the amniotic fluid protein (Fig. 2, lane 3), of several bands in the anodic portion of the gel that are very similar to those of the urinary protein (Fig. 2, lane 1).

Reaction with 3-OHKyn—It is known that under physiological conditions Kyn is converted into 3-OHKyn, an o-aminophenol, which is its most abundant metabolite in biological fluids (28). It has also been shown that 3-OHKyn reacts with lens proteins and that this covalent attachment is associated with the formation of tanned products (29). The coloration has been attributed to the formation of autoxidation derivatives which display absorbance maxima between 350 and 490 nm (21). Therefore, amniotic fluid α-1-m and HSA were incubated with 3-OHKyn to allow the formation of the amniotic to yield the α,β-unsaturated ketone occurs relatively slowly (20), compared with the oxidation of 3-OHKyn in the presence of oxygen (21). Thus, the incubation was carried out at pH 9.5, for Kyn, to accelerate the reaction with the proteins. Following this incubation (Fig. 3B, line 3), longer wavelength-absorbing species were present in the amniotic fluid α-1-m samples, as well as in those of HSA (not shown), and both spectra became much more similar to that of the urinary lipocalin. The fluorescence spectra of the amniotic lipocalin prior to and after incubation with 3-OHKyn displayed the same emission maxima in the spectral region of 455–460 nm observed after the incubation with Kyn. These results show that upon reaction with 3-OHKyn in the presence of oxygen the light yellow amniotic α-1-m acquires the characteristic absorbance of the urinary lipocalin in the 350- to 500-nm range, thus suggesting that the heterogeneous modification of the latter is due to the oxidation products of the 3-OHKyn-tREATED amniotic fluid protein (Fig. 2, lane 3), of several bands in the anodic portion of the gel that are very similar to those of the urinary protein (Fig. 2, lane 1).

The HPLC trace and the ESI/MS data obtained after acid hydrolysis of urinary α-1-m were then compared with those of HSA that was hydrolyzed following incubation with 3-OHKyn (M, 224). As observed for the urinary lipocalin, the HPLC trace of the modified albumin (Fig. 6A, line b) shows several colored peaks that were separated, collected, and submitted to LC-MS. In addition, to identify unequivocally the molecular ions attributable to adducts with 3-OHKyn and its oxidized derivatives, the total hydrolysates obtained from the modified HSA and from the urinary lipocalin were compared by LC-MS with that of the normal serum protein (data not shown). The mass spectra of the major colored peaks of the urinary lipocalin and of the modified HSA have charged species in the range 200–600 that are not present in the albumin control. In particular, the species yielding the ion at m/z 389 [M+H]+ (Fig. 8) was detected also under peak 2 in the hydrolysate of modified HSA after RP-HPLC separation (Fig. 6A, line b). Its fragmentation pattern was identical to those of the m/z 389 ions observed in the spectra of urinary and amniotic fluid α-1-m (Fig. 9). In addition, two charged species at m/z 555 and at m/z 517, which elute under peaks 4’ and 5’, respectively, in the HPLC trace of the urinary lipocalin (Fig. 6B), are present also in the spectra of peaks 4 and 5 from modified HSA (Fig. 6A, line b) and were submitted to MS/MS. Fig. 10 shows the LC-MS chromatograms and the tandem mass spectra of the singly charged ions at m/z 555 [M+H]+ from urinary α-1-m (panel A) and from modified albumin (panel B). Both spectra displayed the ion fragment at m/z 409, due to the loss of lysine, [M+H-Lys]+, and the charged species at m/z 391 [M+H-Lys-H2O]+ following the loss of water. The molecular ions at m/z 517 [M+H]+ from the two
proteins showed the same fragmentation pattern with product ions at m/z 371 [M+H-Lys]+, and m/z 353 [M+H-Lys-H2O]+ (data not shown). The observed m/z values were compared with the monoisotopic m/z values corresponding to the putative modification of either the Lys or His residues upon reaction with the known autoxidation derivatives of 3-OHKyn. Only the molecular ion at m/z 555 was identified as the [M+H]+ ion of the Lys-HXan adduct. The MS/MS spectra of the molecular ion at m/z 555 obtained for α-1-m and modified HSA were identical and showed the presence of product ions indicative of the identified adduct, including a diagnostically charged species at m/z 409 for the protonated and deaminated form of HXan. The ion at m/z 517 is a Lys adduct and, although its major product ion at m/z 371, due to the elimination of the amino acid, could not be identified, its presence in both spectra proves that it is a 3-OHKyn derivative.

Because other charged species attributable to adducts were detected but could not be identified, the 3-OHKyn adducts of N-acetyl-Lys and N-acetyl-His were prepared, in the presence of oxygen, and, after deprotection of the amino group, separated by RP-HPLC. The traces show the presence of several colored peaks (data not shown) that were collected and analyzed by LC-MS/MS. The 3-OHKyn-Lys adduct at m/z 354 was found and showed the expected fragment ions, including the m/z 203 charged species indicative of the attachment site. In addition lysine yielded several other derivatives that gave MS/MS spectra attributable to different reaction mechanisms. The yield of the 3-OHKyn-His adduct at m/z 354 [M+H-H2O]+...
was very low, whereas the His adduct with Xan (M, 423) at m/z 562 [M+H]+ was clearly identified and showed ion fragments at m/z 407 [M+H-His-NH2]+ and m/z 388 [M+H-His-NH2-H2O]+ that differ by two mass units from the fragment ions of the charged species at m/z 555. HXan (M, 425) represents the major product formed by the oxidative dimerization of 3-OHKyn, and the same Lys adduct is present in the hydrolysate of serum albumin following reaction with the metabolite in the presence of oxygen. These results show that the lipocalin is linked to 3-OHKyn derivatives and indicate that the polycyclic HXan is one of the chromophores covalently bound to the urinary protein. Other present molecular ions such as the charged species at m/z 389, that were not identified, appear to agree with the view that 3-OHKyn not only reacts via the α,β unsaturated ketone, but it is also the precursor of a series of oxidative derivatives that can form different types of adducts with proteins.

**DISCUSSION**

HXan, together with Xan, represents the major autoxidation product of 3-OHKyn under physiological conditions, and the results presented here show that it is one of the chromophores covalently linked to α-1-m isolated from urine of hemodialyzed patients. The Lys-HXan adduct was isolated from the lipocalin hydrolysate and characterized by tandem mass spectral analysis. It was also identified in a sample of serum albumin, previously incubated with 3-OHKyn, thus showing that the modification is due to reaction with the metabolite. The products of tryptophan degradation, which display absorbance maxima in the visible range that are very similar to the characteristic UV-visible spectrum of lipocalin, are responsible for the peculiar yellow-brown color of the protein. This reaction appears also to be the cause of the charge heterogeneity of the urinary protein observed in isoelectric focusing. Color and charge heterogeneity are significantly less pronounced in the light yellow amniotic fluid protein but can be induced by incubation with 3-OHKyn in the presence of oxygen. The linkage to the polypeptide chain was found to involve lysyl residues, but it is known from other studies that 3-OHKyn can also react with histidine and cysteine (16). The fact that no His-adducts were found in the hydrolysate of the urinary protein probably reflects its lower abundance in the α-1-m polypeptide chain, which contains 11 Lys and only 4 His residues. Several factors, including the adduct stability at physiological pH, the three-dimensional structure of the protein, the residue accessibility, and their involvement in salt bridges (30), likely influence the extent of the modification. Furthermore, comparison of the m/z values of the ions in the hydroxylated lipocalin spectrum with those present in the hydroxylated native and modified albumin shows that the lysines of the latter react very extensively with Kyn. Examination of the tandem mass spectra of other adducts that could not be identified in the hydrolysates suggests that they are formed through different reaction mechanisms.

Although the exact identity of the chromophores bound to α-1-m was unknown, it has been shown that one modification involved the cysteine residue at position 34 and that the link was through a reduction resistant bond (11). This amino acid is expected to react with 3-OHKyn, but, in addition to the inherent instability at neutral pH of the adduct compared with those with His and Lys (15), only one free Cys is present in α-1-m and therefore the relative amount of the derived species would be too low to make it detectable after acid hydrolysis. More recently, three lysyl residues (lysines 92, 118, and 130) surrounding the entrance of the lipocalin pocket (12) were identified as the modified amino acids, following tryptic cleavage of the polypeptide chain, although the nature of the covalently bound chromophores was not established. The colored, heterogeneous in size tryptic peptides appeared to have a tendency to aggregate and form complexes, which is in agreement with the results presented here (12).

All earlier investigations report to have observed appreciable light absorbance in the visible region of α-1-m isolated from any source (10). However, we have found that, although the protein purified from the urine of hemodialyzed individuals, which represents the most common source of α-1-m, displays the typical intense yellow-brown color, the lipocalin from normal amniotic fluid, which contains the fetal urinary proteins, is only slightly yellow. In addition, when examined by isoelectric focusing and SDS-PAGE electrophoresis, the latter is less heterogeneous in charge and size. The two forms of the protein have been well characterized, and their amino acid and oligosaccharide structures can not be correlated with the observed optical and electrophoretic differences, which, instead, appear attributable to the covalently linked chromophores. Following the incubation with 3-OHKyn under oxidative conditions the amniotic fluid protein acquired the long wavelength absorption characteristics of the urinary α-1-m thus indicating that these properties are due to the presence of oxidative products that are formed in much lower amounts in the normal amniotic fluid. Therefore, the more intense tanning of the urinary protein appears to be attributable to the condition of uremia and hemodialysis. Similar to other small plasma proteins, α-1-m is mainly eliminated in the kidneys by glomerular filtration followed by tubular reabsorption and catabolism. Therefore, disorders associated with a decreased glomerular filtration rate cause a rise in the protein levels in serum from the normal value of 50 to up to 300 μg/liter. Several reports made it evident that in renal disease the tryptophan catabolism via the kynurenine pathway is increased and is accompanied by the rise in the concentration of its derivatives in blood and in peripheral tissues in proportion to the severity of renal impairment (31). The level of Kyn in plasma is 1.6 μM in healthy subjects and 2.7 μM in hemodialyzed patients, undergoing treatment, which results in a 10-fold increase in the 3-OHKyn concentration from 39 to 397 nm (13). The activity of the kynurenine pathway enzymes and the distribution of the resulting products in plasma and peripheral tissues have been examined in rats with chronic renal failure (31). The results showed that, in kidneys and in the liver, the activity of the enzyme responsible for the conversion of Kyn to 3-OHKyn, kynurenine-3-hydroxylase, is increased while that of the enzymes involved in its degradation is decreased. Thus, the mechanism of 3-OHKyn accumulation appears to be associated not only with impaired excretion but also with increased synthesis and lower degradation rate. Moreover, the prolonged life of the lipocalin in uremic patients exposes the protein to higher concentrations of these metabolites during longer periods. The characterization of the major autoxidation products of 3-OHKyn under physiological conditions showed that, when exposed to either oxygen or air, the compound dimerizes to HXan and Xan, which, in turn, generate at least five different derivatives (21). In the presence of a catalyst, such as potassium ferricyanide, Xan was reported to be the only oxidation product (23), whereas one electron oxidation of HXan produces a stable HXan free radical with a long lifetime. This reaction can be induced under physiological conditions when 3-OHKyn is incubated with hemoglobin or hemin (23). Thus, the hemodialysis treatment, which is accompanied by the release of hemoglobin and hematin from lysed erythrocytes, is likely to cause the pronounced tanning of the protein due to a more extensive oxidation of 3-OHKyn. This is in agreement with a recent report showing that α-1-m isolated from plasma of healthy subjects, which has a lower absorbance in the near UV and visible region when compared with the
urinary protein, develops the heterogeneous spectrum of the latter after incubation with lysed erythrocytes (32).

There is accumulating evidence suggesting that disturbances in the pathway of tryptophan degradation associated with uremia might have clinical relevance and that the toxic degradation products of kynurenine in hemodialyzed patients contribute to some symptoms such as neurological disturbances and decreased glomerular filtration. In addition, several components of the pathway have been attributed pro- and anti-oxidant properties in vivo contributing to the oxidative stress associated with neurodegenerative disorders and neuronal damage. In the present study we have shown that the covalent modification of α-1-m by the oxidation products of 3-OHkyn produces colored proteins similar to those observed in the nuclear cataract were the metabolite has also been shown to act as a protein cross-linking agent (16). The finding, that upon storage at slightly basic pH the urinary lipocalin forms dimers and higher order oligomers while the amniotic form remains predominantly monomeric strongly, suggests that 3-OHkyn oxidation products are indeed involved in the α-1-m dimerization and possibly in the well known propensity of the lipocalin to produce complexes with other plasma proteins.

Kynurenic derivatives are also implicated in the pathophysiology of a variety of human brain diseases, including human immunodeficiency virus-related dementia (33), Parkinson’s disease (34), and Huntington’s disease (35), and the cytotoxic effect has been ascribed to the oxidative stress caused by these compounds (36, 37). Recent reviews present evidence supporting the role of protein aggregates in the etiology of neurodegenerative disorders (38–40). Therefore, the formation of oligomers of other proteins, like those observed in the lens and in modified α-1-m, in the presence of increased levels of 3-OHkyn could play a role in the pathogenesis of these and other diseases that is worthwhile exploring in detail.

Acknowledgments—We thank Rossella Greco, Department of General Biology and Medical Genetics, University of Pavia, and the Hemodialysis Unit, Salvatore Maugeri Foundation, Pavia, for supplying the biological fluids; Patrizia Arcidiacono for sequence analysis; and Dr. Hugo L. Monaco and Dr. Luigi Casella for critically reading the manuscript.

REFERENCES

1. Flower, D. R., North, A. C., and Sansom, C. E. (2000) Biochim. Biophys. Acta 1452, 9–24
2. Berggard, T., Oury, T. D., Thogersen, I. B., Akerstrom, B., and Enghild, J. J. (1998) J. Histochem. Cytochem. 46, 887–894
3. Tyagi, S., Srjtt, M., Roy, A. K., Jameel, S., and Lai, S. K. (2004) J. Biol. Chem. 279, 29308–29319
4. Kaumeyer, J. F., Polazzi, J. O., and Kotik, M. P. (1986) Nucleic Acids Res. 14, 7839–7850
5. Amoresano, A., Minchiotti, L., Cassilich, M. E., Campagnoli, M., Pacci, P., Andolfi, A., Gianazza, E., and Galliano, M. (2000) Eur. J. Biochem. 267, 2105–2112
6. Salier, J. P. (2000) Biochim. Biophys. Acta 1482, 25–34
7. Loevy, A., Rouet, P., Salier, J. P., and Akerstrom, B. (1999) Gene 234, 329–336
8. Mendez, E., Fernandez-Luna, J. L., Grubb, A., and Leyva-Cobian, F. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1472–1475
9. Grubb, A. (1992) Clin. Nephrol. 38, 820–827
10. Akerstrom, B., Logberg, L., Berggard, T., Osmark, P., and Lindqvist, A. (2000) Biochim. Biophys. Acta 1482, 172–184
11. Eskilson, J., Grubb, A., Calero, M., and Mendez, E. (1991) J. Biol. Chem. 266, 15758–15763
12. Berggard, T., Cohen, A., Persson, P., Lindingqvist, A., Cedervall, T., Silow, M., Thogersen, I. B., Jonsson, J. A., Enghild, J. J., and Akerstrom, B. (1999) Protein Sci. 8, 2611–2620
13. Grubb, A., Gianazza, E., Astrua-Testori, S., Giacon, P., and Righetti, P. G. (1985) Arch. Biochem. Biophys. 249, 616–622
14. Ishii, T., Iwahashi, H., Sugata, R., and Kido, R. (1992) Biochim. Biophys. Acta 1145–1146
15. Galliano, M., Minchiotti, L., Perta, F., Rossi, A., Ferri, G., Madison, J., Watkins, S., and Putnam, F. W. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8721–8725
16. Gianazza, E., Astrua-Testori, S., Giacon, P., and Righetti, P. G. (1985) Electrophoresis 6, 332–339
17. Vazquez, S., Aquilina, J. A., Carver, J. A., Jamie, J. F., Sheil, M. M., and Truscott, R. J. (2000) J. Biol. Chem. 275, 4867–4873
18. Aquilina, J. A., Carver, J. A., and Truscott, R. J. (1999) Biochemistry 38, 11455–11464
19. Asanuma, N., Asanuma, T., and Ikeda, T. (1997) J. Chrom. A 773, 23–31
20. Ishii, T., Iwahashi, H., Sugata, R., and Kido, R. (1992) Arch. Biochem. Biophys. 294, 616–622
21. Vazquez, S., Garner, B., Sheil, M. M., and Truscott, R. J. (2000) J. Biol. Chem. 275, 32547–32550
22. Aquilina, J. A., Carver, J. A., and Truscott, R. J. (1999) J. Biol. Chem. 274, 16176–16184
23. Stone, T. W., and Darlington, L. G. (2002) Nat. Rev. Drug Discov. 1, 609–620
24. Aquilina, J. A., Carver, J. A., and Truscott, R. J. (1999) Biochemistry 38, 16176–16184
25. Mendez, E., Fernandez-Luna, J. L., Grubb, A., and Sheil, M. M. (1991) J. Cell Biol. 114, 2611–2620
26. Okado-Matsumoto, A., and Fridovich, I. (2002) J. Biol. Chem. 277, 11799–11807
27. Schwarcz, R., and Pellicciari, R. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 10010–10014
28. Beaulieu, J. M., Nguyen, M. D., and Julien, J. P. (1999) J. Cell Biol. 147, 531–544