The Lipocalin α₁-Microglobulin Has Radical Scavenging Activity*

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The lipocalin α₁-microglobulin (α₁m) is a 26-kDa glycoprotein present in plasma and in interstitial fluids of all tissues. The protein was recently shown to have reductase properties, reducing heme-proteins and other substrates, and was also reported to be involved in binding and scavenging of heme and tryptophan metabolites. To investigate its possible role as a reductant of organic radicals, we have studied the interaction of α₁m with the synthetic radical, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS radical). The lipocalin readily reacted with the ABTS radical forming reduced ABTS. The apparent rate constant for this reaction was 6.3 ± 2.5 × 10³ M⁻¹ s⁻¹. A second reaction product with an intense purple color and an absorbance maximum at 550 nm was formed at a similar rate. This was shown by liquid chromatography/mass spectrometry to be derived from covalent attachment of a portion of ABTS radical to tyrosine residues on α₁m. The relative yields of reduced ABTS and the purple ABTS derivative bound to α₁m were ~2:1. Both reactions were dependent on the thiolate group of the cysteine residue in position 34 of the α₁m polypeptide. Our results indicate that α₁m is involved in a sequential reduction of ABTS radicals followed by trapping of these radicals by covalent attachment. In combination with the reported physiological properties of the protein, our results suggest that α₁m may be a radical reductant and scavenger in vivo.

The lipocalins are a protein superfamily with 30–35 members distributed among animals, plants, and bacteria (1, 2). The members of the superfamily have a highly conserved three-dimensional structure, 8–9 antiparallel β-strands folded into a barrel with one closed and one open end. The interior of the barrel forms a binding site for small hydrophobic ligands, and this structural property is the basis for a surprisingly wide array of biological functions. So far, three lipocalins have been shown to be enzymes: prostaglandin D-synthase (3), violaxanthin de-epoxyclease in plants (4), and α₁-microglobulin (α₁m)² (5–7), which was recently shown to have reductase/dehydrogenase properties (8).

Also called protein HC (9), α₁m is one of the originally described lipocalins (10) and is one of the most widespread lipocalins phylogenetically (7). So far, it has been found in mammals, birds, fish, and amphibians. The protein is synthesized by the liver (11), rapidly distributed by the blood to the extravascular compartment (12), and found in most organs in interstitial fluids, connective tissue, and basement membranes (13–15). It is especially abundant at interfaces between the cells of the body and the environment, such as in lungs, intestine, kidneys, and placenta (16–18). Due to its small size, 26 kDa, α₁m is rapidly cleared from the blood by glomerular filtration. Most of the filtered α₁m is degraded in the kidneys, but a small part is excreted in the urine (12). α₁m isolated from plasma and urine is yellow-brown and displays charge heterogeneity (i.e., a broad band upon electrophoresis) (19). This is caused by an array of small chromophoric groups attached to the amino acid residues Cys-34, Lys-92, Lys-118, and Lys-130, which are localized around the entrance of the lipocalin pocket (20–22). The biological function of α₁m is unknown, although it has a number of immunosuppressive properties, such as inhibition of antigen-induced lymphocyte cell proliferation, cytokine secretion (23–25), and the oxidative burst of neutrophils (26). Several recent findings suggest that α₁m is involved in reduction and scavenging of biological pro-oxidants, such as heme and heme-proteins. First, it was shown that α₁m binds heme strongly and obtains the yellow-brown chromophore by incubation with hemoglobin or erythrocyte ghosts, concomitant with degradation of the bound heme (27). A processed form, t-α₁m, which lacks the C-terminal tetrapeptide LIPR and has enhanced heme degradation properties, is also induced by incubation with hemoglobin. t-α₁m is found in urine (27) and continuously forms in chronic leg ulcers, a hemolytic inflammatory condition where free heme and iron are considered to be oxidative pathogenic factors (28). Second, lysyl residues in urine α₁m from hemodialysis patients were found to be modified by kynurenine derivatives (29). These are tryptophan catabolites that have a propensity to form free radicals (30–32) and are present at elevated concentrations in plasma of hemodialysis patients.

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² The abbreviations used are: α₁m, α₁-microglobulin; ABTS, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); HPLC, high pressure liquid chromatography; HSA, human serum albumin; IAA, iodoacetamide; LC, liquid chromatography; MS, mass spectrometry; PBS, phosphate-buffered saline.
patients (33). Fourth, α₄m was shown to enzymatically reduce cytochrome c, methemoglobin, nitro blue tetrazolium, and free iron, using NADH, NADPH, or ascorbate as electron-donating co-factors (8). The thiol group in position Cys-34 and the three lysyls Lys-92, Lys-118, and Lys-130 were implicated in the active site. Finally, the cellular expression of α₄m is up-regulated by hemoglobin and radical oxygen species (34).

These reports suggest that α₄m could potentially undergo reactions with biological radicals and that these reactions may be related to its physiological function. To investigate how α₄m reacts with radicals, we have studied the interaction of α₄m with the stable radical, 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). This compound has been used extensively to investigate antioxidant mechanisms (e.g. see Ref. 35). We show that α₄m reduces the ABTS radical and simultaneously covalently binds to the radical, forming a distinct purple adduct. The results suggest that the lipocalin α₄m may be a radical reductase and scavenger in vivo.

MATERIALS AND METHODS

Proteins and Reagents—Wild type and mutated variants of α₄m were expressed in Escherichia coli. Using site-directed mutagenesis, a Cys → Ser substitution was introduced at amino acid position 34 to give the C34S-α₄m mutant (36). In the recombinant α₄m forms, the N terminus was elongated by a 15-amino acid peptide containing eight histidines (His tag) and an enterokinase cleavage site (DDDDK). The His tag was removed by incubating 10 mg of α₄m with 400 units of enterokinase (Sigma) for 5 h at room temperature in 20 mM Tris-HCl, 0.5 mM NaCl, pH 8.0. His tag-free α₄m was then separated from enterokinase by gel chromatography, and the N-terminal amino acid sequence was determined.

Human α₄m was prepared from plasma (37), urine (38), and baculovirus-infected insect cells (39) as described. Human plasma, urine, salivary, and tear fluid were obtained from healthy volunteers. All other proteins and reagents were of analytical grade and were purchased from Sigma if not indicated otherwise.

Alkylation of α₄m—Thiol groups were alkylated by incubating α₄m (0.18 mM) with 22 mM iodoacetamide (IAA) in phosphate-buffered saline (PBS; 10 mM sodium phosphate, pH 7.4, 120 mM NaCl, 3 mM KCl) for 1 h at room temperature in the dark and then dialyzing exhaustively against 25 mM Tris-HCl + 50 mM NaCl, pH 8. Reduced thiol groups were quantified by alkylation with iodo-[^14]C]acetamide ([^14]C]IAA) (Amersham Biosciences; specific activity 59.0 mCi (2.2 GBq)/mmol). The reaction mixtures contained 2 μM α₄m in 0.2 mM Tris-HCl, pH 8.5, and 1 mM [^14]C]IAA. The reaction proceeded for 60 min at 25°C in the dark. To determine the amounts of bound [^14]C]IAA, the alkylated α₄m was subjected to SDS-PAGE and phosphorimaging.

Reduction of ABTS Radical—A stock solution of ABTS radical was prepared following the procedure of Re et al. (40) with minor modifications. Potassium persulfate was added to a 7 mM ABTS solution in water, to a final concentration of 2.8 mM, allowing at least 5 h for the reaction. The solution was kept in the dark and used within 24 h. In some experiments, the amount of potassium persulfate was varied, producing different ABTS radical/ABTS ratios. The stock solution was diluted 125 times with PBS. α₄m, control proteins, and other reagents were added as described for each experiment, and the reaction was followed by monitoring absorbance changes of ABTS and its radical. End point scanning of the reaction products was done after reducing remaining ABTS radicals by adding NaNO₂ to a final concentration of 60 mM as described (41). pH studies were done by diluting the stock solution with 20 mM sodium acetate, pH 5.0, or 20 mM sodium phosphate, pH 6.0, 7.0, or 8.0, or 20 mM glycine-OH, pH 9.0.

Estimation of Kinetic Parameters—Initial reaction rates were estimated by linear regression analysis of absorbance values obtained during the first 1 min of the reaction. The total formation of products (i.e. the reduced form of ABTS and the purple α₄m modification) and the total consumption of ABTS radical during the initial, rapid reaction phase were determined by linear regression analysis, as illustrated in Fig. 3. Kₘ and Vₘₐₓ values were determined by nonlinear regression of initial reaction rates using different initial α₄m (0.5–4 μM) and ABTS radical (7–55 μM) concentrations and using the same ratio between the initial ABTS radical and ABTS concentrations.

Spectrophotometric Determinations—Spectrophotometric analyses were done either in a Beckman 7500 photodiode array spectrophotometer or a Beckman DU 640i spectrophotometer. The reaction between α₄m and ABTS radical was followed by scanning a 0.5-ml reaction mixture, blanking with PBS or the appropriate dilution buffer. Reading at time 0 was done before the addition of α₄m or control proteins, and at regular time intervals after the addition of the proteins. Concentrations of ABTS were determined by using ε₅₅₀ = 4.8 × 10^5 M⁻¹ cm⁻¹ and of the ABTS radical using ε₅₅₀ = 3.6 × 10^4 M⁻¹ cm⁻¹ (42). Concentrations of α₄m were determined by using the extinction coefficients at 280 nm reported for urine, plasma, and baculovirus-infected insect cell α₄m (39) and 3.6 × 10^4 M⁻¹ cm⁻¹ for recombinant α₄m. The absorbance values at 550 nm at different time points (A₅₅₀) were calculated after correction for spillover absorbance of the ABTS radical using the following formula: A₅₅₀ = 0.5 × (A₅₅₀(observed) − 0.403 × A₇₃₅) + 0.5 × (A₅₅₀(observed) − 0.163 × A₆₉₅). The coefficients in this equation were determined by absorbance scanning of the ABTS radical at known concentrations.

Purification of Purple α₄m—The purple end product of α₄m was purified by gel filtration. α₄m (80–140 μM) was incubated for 5 min with the ABTS radical/ABTS stock solution (1.2–1.8 mM) in PBS. After centrifugation at 8000 × g for 2 min, the reaction product was applied to a 1-ml column packed with Sephadex G-25 Fine and equilibrated with 2 mM NH₄HCO₃, pH 8.5. The column was eluted with the equilibration buffer at flow, and 0.2-ml fractions were collected manually. The eluted fractions were analyzed by absorbance scanning. Protein-containing fractions were pooled.

SDS-PAGE, Blotting, and N-terminal Sequence Analysis—SDS-PAGE was performed using 12% gels in the buffer system described by Laemmli (43), with or without 2% (v/v) β-mercaptoethanol in the sample buffers. High molecular mass standards (Rainbow markers; Amersham Biosciences) were used. N-terminal amino acid sequence analysis was achieved by Edman degradation (Protein Analysis Center, KL, Stockholm, Sweden).
of bands separated by SDS-PAGE and transfer to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) as described (44).

Reaction of ABTS Radical with Tyrosine—ABTS radicals were generated by using hydrogen peroxide (50 μM) and lactoperoxidase (5 μg/ml) to oxidize ABTS (100 μM) in 50 mM phosphate buffer, pH 7.4. When the formation of ABTS radicals reached a maximum (approximately 35 μM), catalase (10 μg/ml) was added to scavenge residual hydrogen peroxide. Tyrosine (10–30 μM) was then reacted with the ABTS radicals. It caused a stoichiometric loss in ABTS radicals within 5 min and promoted the formation of a product that had an absorbance maximum from 500 to 550 nm. This final reaction mixture was analyzed by LC/MS as described below.

Reaction of α₉m with Glycyl-Tyrosyl Radicals—Radicals of the Gly-Tyr peptide were generated using lactoperoxidase and hydrogen peroxide. Reactions were carried out in 10 mM phosphate buffer, pH 7.8, containing 140 mM NaCl, 20 μM Gly-Tyr, 25 μg/ml lactoperoxidase, and 10 μM diethylenetriaminepentaacetic acid with or without α₉m, orosomucoid, or HSA. They were started by adding 5 μM hydrogen peroxide. After 30 min at 20 °C, the fluorescence due to formation of dityrosine was measured (λ_ex, 325 nm; λ_em, 405 nm) in a Jasco J-810 Spectrofluorimeter (Jasco Scandinavia AB, Mölndal, Sweden).

Preparation of Protease Fragments of α₉m after Reaction with ABTS Radicals—ABTS and α₉m were reacted and desalted as described previously to generate the purple product. After boiling for 2 min, Pronase (Protease XIV from Streptomyces griseus) at a 1:25 enzyme/substrate ratio was added to the ABTS–α₉m and incubated at 37 °C for 2 h. Alternatively, trypsin was added at a ratio of 1:10 and incubated at 37 °C for 3 h. The digests were then subjected to HPLC separation on a Phenomenex Luna 5 μC18 column (250 × 4.6 mm) using the following stepwise gradient. From 0 to 5 min, the eluent was 100% solvent A (10% methanol in 50 mM phosphate buffer, pH 6.5, containing 2.5 mM 3-octylamine); the organic phase was then increased to 50% over the following 5 min and kept at 50% between 10 and 30 min. The flow rate was 0.8 ml/min. Peaks that absorbed at 550 nm were well resolved from ABTS and ABTS radical. They were subjected to HPLC separation on a Phenomenex Luna 5 μC18 column (250 × 4.6 mm) using the following stepwise gradient. From 0 to 5 min, the eluent was 100% solvent A (10% methanol in 50 mM phosphate buffer, pH 6.5, containing 2.5 mM 3-octylamine); the organic phase was then increased to 50% over the following 5 min and kept at 50% between 10 and 30 min. The flow rate was 0.8 ml/min. Peaks that absorbed at 550 nm were well resolved from ABTS and ABTS radical. They were analyzed by LC/MS as described below.

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Liquid Chromatography-Mass Spectrometry Analysis of α₉m Digests—Tryptic peptides were analyzed by LC/MS by selecting and fragmenting major ions that eluted in the chromatogram. Peptides that contained ABTS were identified by the presence of an absorbance maximum around 550 nm and characteristic molecular fragments of ABTS in the MS/MS spectrum. The purple digestion products of the ABTS–α₉m were separated on a Jupiter Proteo column (particle size 4 μm, 150 × 2.0 mm) using a Surveyor HPLC pump (Thermo Corp., San Jose, CA). The column was maintained at 30 °C. The products were eluted at a flow rate of 0.2 ml/min using a linear gradient of two solvents: solvent A (0.1% formic acid) and solvent B (0.1% formic acid, 90% acetonitrile). The gradient was as follows: 0–30 min, increased solvent B to 50%; 30–32 min, increased solvent B to 100%; 32–34 min, maintained solvent B at 100%; 34–35 min, decreased solvent B to 0%. The injection volume was 20 μl. The HPLC was coupled to an ion trap mass spectrometer (ThermoFinnigan LCQ Deca XP Plus; Thermo Corp.) equipped with an electrospray ionization source. The mass spectrometer was operated with positive ionization using full scan mode (scan range 100–2000 m/z). Spray voltage was set at 3.5 kV, the capillary temperature was set at 275 °C, and the sheath gas flow was set at 35 units (instrument units). For MS/MS experiments, parent ions were fragmented in the ion trap using 35% collision-induced energy.

RESULTS

Reaction between α₉m and ABTS Radical—The ABTS radical was readily reduced by α₉m (Fig. 1). Overlaid scans of the solution show a decrease of the ABTS radical-specific 415 and 735 nm peaks and a concomitant increase of the ABTS-specific peak at 340 nm. An almost complete reduction was seen after...
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The pH dependence of the reactions was investigated between pH 5 and 9 using standard conditions (Fig. 2C). A slow rate of ABTS radical consumption and formation of reduced ABTS was seen at pH 5, the rates increased between pH 5 and 8, and no further increase was seen at pH 9. This suggests possible involvement of cysteine, tyrosine, or histidine residues on α1m, side chains on which deprotonization is likely to occur between pH 5 and 8. A much more striking pH dependence was seen for the formation of the purple ABTS-α1m. The absence of reaction below pH 7 and a sharp increase in the rate between pH 7

20 min by 3.5 μM α1m (Fig. 1A). Remaining ABTS radicals were reduced by adding 60 mM NaN3, revealing two end products, reduced ABTS, represented by the 340 nm peak, and a novel peak at 550 nm (Fig. 1A, inset), which gave the solution a purple color. Two separate phases of the reaction could be distinguished (Fig. 1B), an initial faster phase over the first 5 min and a second slower phase that was still ongoing after 2 h. A clear difference in the rate of the first phase was seen between α1m and HSA (Fig. 1B) as well as the control proteins ovalbumin, orosomucoid, and soybean trypsin inhibitor (not shown). In contrast, the rate of the second phase was similar between α1m and the control proteins. Thus, the second phase was regarded as nonspecific and is not discussed further. After 5 min, ~8–9 ABTS radical molecules had been consumed per molecule of α1m. Trolox, a water-soluble analogue of vitamin E, reduced ABTS radical stoichiometrically at a 1:1 molar ratio (not shown).

To study the reaction products, 1 mg of α1m was allowed to react with a ~10–12-fold excess of ABTS radicals for 5 min, applied to a Sephadex G-25 column, and eluted, and fractions were collected (Fig. 2, A and B). The purple product (i.e. the absorbance at 550 nm) co-eluted with the α1m protein (absorbance at 280 nm), whereas remaining ABTS radicals and ABTS were eluted later.

![Image](https://via.placeholder.com/150)

FIGURE 2. Preparation of ABTS-α1m (A and B) and pH dependence of the reaction between α1m and ABTS radical (C). α1m was added (final concentration 0.14 mM) to ABTS radical (1.7 mM) and ABTS (0.8 mM) in 60 mM Tris-HCl, pH 8, 0.5 mM NaCl and incubated for 5 min. To illustrate the separation, fresh ABTS radical was then added to 1 μM A, the reaction mixture was applied to a 2-ml column packed with Sephadex G-25 Fine and eluted with 20 mM NH4HCO3, pH 8.5. The positions of the green ABTS radical and the purple ABTS-α1m are indicated by arrows. B, fractions were collected and analyzed by reading the absorbance spectrum. The absorbance values at 280 nm ( ), 340 nm ( ), 415 nm ( ), and 10× 550 nm ( ) of each fraction were plotted. C, the reaction of 2 μM α1m with the standard ABTS radical/ABTS solution was followed at various pH values by monitoring the absorbance at 340, 415, and 550 nm over 20 min. The initial rates of ABTS radical consumption ( ) and formation of reduced ABTS, represented by the 340 nm peak, and a novel green product ( ), and formation of purple product ( ) of each fraction were plotted.

| Medium          | Initial rate of ABTS radical loss* | Reaction rate (μM/s) |
|-----------------|----------------------------------|----------------------|
|                 | 50×c                           | 100×c            | 200×c          |
| Buffer          | 0.37 (0.02)d                    | 0.36 (0.03)        | 0.37 (0.05)    |
| Plasma          | 0.34 (0.05)                     | 0.35 (0.04)        | 0.36 (0.03)    |
| Urine           | 0.33 (0.04)                     | 0.43 (0.04)        | 0.41 (0.03)    |
| Saliva          | 0.38 (0.03)                     | 0.37 (0.03)        | 0.36 (0.04)    |

* Calculated as described under “Materials and Methods,” subtracting the rate of ABTS radical loss in the absence of α1m.

# Table 1

Reaction rates in various physiological media of 2.5 μM α1m and the standard ABTS radical/ABTS solution

![Image](https://via.placeholder.com/150)

FIGURE 3. Kinetics of the reactions between α1m and ABTS radical. A, the reaction of 1 μM α1m with the standard ABTS radical/ABTS solution was followed by reading the absorbance and the concentration of the ABTS radical, ABTS, and A550. B, the net loss of ABTS radical was plotted from the experiment in A. The initial reaction rate and end point ABTS radical consumption during the initial rapid reaction phase were estimated as shown in the figure.
Influence of the Cys-34 thiol group of $\alpha_1$m on the reaction with ABTS radical. The reaction of 2 $\mu$M wild type (wt), C34S, alkylated (IAA) $\alpha_1$m, or HSA with the standard ABTS radical/ABTS solution was followed by reading the absorbance spectra at different time points. The initial rates of the ABTS radical consumption, ABTS formation, and absorbance at 550 nm were calculated as described in the legend to Fig. 5B. Each bar represents the mean $\pm$ S.D. of triplicate experiments.

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and 8 suggest involvement of side groups with a $pK_a$ around 7.5. The His tag of the recombinant $\alpha_1$m did not influence the rates of ABTS radical consumption or formation of ABTS-$\alpha_1$m (not shown). This was supported by experiments using plasma and urine $\alpha_1$m, which lack the His tag (see below). The addition of 0.25–1 $\mu$M superoxide dismutase did not slow down the consumption of the ABTS radical or the formation of ABTS-$\alpha_1$m (not shown), suggesting that superoxide radicals were not involved.

The rate of ABTS radical loss during the first 60 s of the reaction was estimated in various body fluids (Table 1). The decay of the radical in the absence of $\alpha_1$m was substantial in most fluids, but at higher dilutions, this could be subtracted from the loss induced by $\alpha_1$m. As shown in Table 1, similar rates are seen in plasma, urine, saliva, and tear fluid, suggesting that similar reactions may occur in vivo. Furthermore, similar rates are seen at various dilutions of saliva and tear fluid.

Kinetics and Stoichiometry—The formation of ABTS-$\alpha_1$m coincided in time with the consumption of ABTS radicals and formation of reduced ABTS (Fig. 3A). This suggests that the three reactions are linked. Fig. 3B illustrates how the rate of the consumption of the ABTS radical and the total consumption of ABTS radical were calculated. The same method was employed to calculate the corresponding parameters for the formation of reduced ABTS and the purple ABTS-$\alpha_1$m.

The reaction rates for ABTS radical consumption, ABTS formation, and production of ABTS-$\alpha_1$m ($A_{550}$), calculated as described in the legend to Fig. 3B, were determined at different initial concentrations of the ABTS radical and ABTS. The rate of formation of ABTS-$\alpha_1$m ($A_{550}$) was independent of the initial ABTS radical and ABTS concentrations (not shown). However, both the rate of ABTS radical consumption and rate of ABTS formation increased to a maximum with increasing initial concentration of ABTS radical (Fig. 4A). The plots show that the rate of consumption of ABTS radical exceeded the rate of formation of reduced ABTS. The $V_{\text{max}}$ and $K_m$ values for the loss of ABTS radicals were calculated by nonlinear regression to be 0.68 $\pm$ 0.06 $\mu$M s$^{-1}$ and 27.2 $\pm$ 8.1 $\mu$M, respectively. These gave a first order rate constant ($V_{\text{max}}/K_m$) for reaction of ABTS radical with $\alpha_1$m of 0.17 $\pm$ 0.02 s$^{-1}$ and an apparent second order rate constant ($V_{\text{max}}/K_m$ or $k_{\text{app}}$) for reduction of ABTS radicals of $6.3 \times 10^3$ M$^{-1}$ s$^{-1}$.

The total ABTS radical consumption and total ABTS formation were plotted as functions of the initial ABTS radical concentration (Fig. 4B). This demonstrated that 8–9 ABTS radical molecules were consumed per $\alpha_1$m molecule, and six ABTS molecules were formed during the first phase of the reaction. This also suggests that up to 2–3 molecules of ABTS were bound to each molecule of $\alpha_1$m (i.e. the amount of ABTS radicals not converted to reduced ABTS).
Reactions with Various $\alpha_{1m}$ Forms and Mutants—The reactions of recombinant $\alpha_{1m}$ purified from human plasma and urine and recombinant $\alpha_{1m}$ from baculovirus-infected insect cells. No significant differences in any of the reaction rates were found between the $\alpha_{1m}$ forms (not shown). The influence of the Cys-34 thiol group was studied using the mutated $\alpha_{1m}$ variant $C34S-\alpha_{1m}$ and alkylated $\alpha_{1m}$ (IAA-$\alpha_{1m}$) (Fig. 5). A significant, but incomplete, decrease of the reduction rate of ABTS radical was seen between the wild type protein and the two thiol group-modified variants. The formation of the purple ABTS-$\alpha_{1m}$ was decreased to background levels using C34S- and IAA-$\alpha_{1m}$. This suggests that the Cys-34 thiol group is essential for the binding of ABTS to the protein and is also involved in the reduction of the ABTS radical.

Molecular Characterization of ABTS-$\alpha_{1m}$—The purified and desalted ABTS-$\alpha_{1m}$ was allowed to react with a solution of ABTS radical (Fig. 6A). The ABTS-$\alpha_{1m}$ reduced the ABTS radical at a decreased rate compared with $\alpha_{1m}$. No more purple color was formed. This suggests that the binding of ABTS on the $\alpha_{1m}$ molecule is saturable (i.e., only a limited number of positions on $\alpha_{1m}$ can be modified) and that the sites for the two reactions are partially linked.

SDS-PAGE shows two major molecular forms of ABTS-$\alpha_{1m}$, 28 and 24 kDa, and the presence of small amounts of a higher molecular weight band (Fig. 6B). A large aggregate was seen in both unreacted wild type $\alpha_{1m}$ and ABTS-$\alpha_{1m}$. The same pat-
tern was obtained without reducing agents in the gel (not shown). The N-terminal sequence of the 24-kDa band was AGPVPT, corresponding to the native protein without the N-terminal His8 tag and enterokinase cleavage site, but with an extra N-terminal alanine. Alkylation of the Cys-34 residue with radiolabeled iodoacetamide showed incorporation to wild type \( \alpha_{1m} \) but not to ABTS-\( \alpha_{1m} \) (Fig. 6B). As expected, C34S-\( \alpha_{1m} \) was negative. This demonstrates that the thiol group of ABTS-\( \alpha_{1m} \) was completely modified. The purple color of ABTS-\( \alpha_{1m} \) could be reduced by a large excess of dithiothreitol, and no spectral evidence of any reduced ABTS or ABTS radical was obtained (not shown). This suggests that the purple product is an oxidized form of ABTS and is covalently linked to the protein moiety.

Identification of Purple Modifications on ABTS-\( \alpha_{1m} \)—Pro

nase digestion of ABTS-\( \alpha_{1m} \) and subsequent purification by HPLC gave two peaks with absorbance at approximately 550 nm. The major peak (Fig. 7, A and B) had a dominant ion of 451 m/z (Fig. 7C). Its MS/MS spectrum (Fig. 7D) had three molecular fragments in common with those of the mass spectrum of the ABTS radical (Fig. 7E). The structure of ABTS and these three fragments are shown in Fig. 9, A and B. The results of this experiment confirm that the purple product contained ABTS or a part of the ABTS molecule.

It has been shown that phenols react with ABTS radicals to form purple compounds with broad absorbance around 550 nm (40, 44). Therefore, we reacted tyrosine with ABTS radical and analyzed the resulting reaction mixture by LC/MS (Fig. 8). It contained a species with a mass of 451 m/z that had an absorbance maximum at 555 nm (Fig. 8, A and B). The mass spectrum of this compound contained three molecular fragments (Fig. 8C) in common with the ABTS radical (Fig. 7E) and the purple product obtained from ABTS-\( \alpha_{1m} \) (Fig. 7D). These results indicate that ABTS radicals react with tyrosyl residues on \( \alpha_{1m} \) to form the purple product that has a mass of 451 m/z. Based on these results and the MS/MS spectra, a structure for the ABTS-Tyr adduct is shown in Fig. 9C.

Several tryptic peptides were identified that absorbed at 550 nm. Two of these could be matched to expected tryptic peptides of unmodified \( \alpha_{1m} \). These two peptides were present in the first fraction from the initial HPLC purification, and they eluted at 13.5 and 14.2 min after subsequent separation by LC/MS (Fig. 10, A and B). They had major ions with m/z ratios of 375.2 (Fig. 10C) and 389.1 mass units (Fig. 10D), respectively. The mass spectra also contained ions with m/z ratios of 749.1 (Fig. 10C) and 777.1 (Fig. 10D) mass units, respectively. These respective ions had the correct m/z ratios to...
indicate that the major ions were a doubly charged species. Thus, the masses of the ABTS-containing peptides were 749.1 and 777.1 mass units.

We calculated masses for the unmodified peptides that form ABTS adducts by subtracting 269 mass units from the singly charged peptides. The value of 269 \(m/z\) was obtained from the ABTS part of the ABTS-Tyr adduct (271 \(m/z\)) minus two mass units that account for the oxidized tyrosine residue. The calculated masses for the unmodified peptide were 480.1 and 508.1 mass units, respectively. These masses correspond to the predicted tryptic peptides IYGK (480.3) and LYGR (508.3), corresponding to tyrosine residues Tyr-22 and Tyr-132, respectively.

Confirmatory evidence that supports these assignments was the presence of ions with \(m/z\) ratios of 478.1 (Fig. 10E) and 506.3 (Fig. 10F) in the respective MS/MS spectra of the doubly charged species. These fragments would arise from the loss of the ABTS portion of the modified peptides, which would give ions 2 mass units less than the unmodified peptides due to oxidation of the tyrosine residues. The presence of ions with \(m/z\) ratios of 244.1 and 259.1 mass units confirms that a portion of ABTS was present in the peptides (cf. Fig. 7). From this finding, it is apparent that ABTS reacts with at least two tyrosine residues in \(\alpha_1m\) to form covalent adducts.

**Reaction of \(\alpha_1m\) with Glycyl-Tyrosyl Radicals**—We determined whether \(\alpha_1m\) can react with other physiologically relevant free radicals. Radicals of the dipeptide Gly-Tyr were generated using lactoperoxidase and hydrogen peroxide (45). Upon adding hydrogen peroxide to the dipeptide and lactoperoxidase, fluorescence associated with dityrosine-like products was produced and was prevented by \(\alpha_1m\) in a concentration-dependent manner (Fig. 11). Neither human serum albumin nor orosomucoid inhibited the fluorescence. Thus, we conclude that \(\alpha_1m\) reacts with a transient oxidant formed during oxidation of Gly-Tyr and that this reaction is not a general activity of proteins.

**DISCUSSION**

In this investigation, we have demonstrated that the lipocalin \(\alpha_1m\) rapidly reacts with the ABTS radical by reduction and covalent binding of ABTS derivatives to tyrosine residues in its polypeptide chain. This activity is superstoichiometric, because one molecule of \(\alpha_1m\) was capable of scavenging 8–9 molecules of the ABTS radical. Furthermore, the rate of reduction of ABTS radical by \(\alpha_1m\) displayed saturation kinetics, which indicates that the ABTS radical must bind to \(\alpha_1m\) before it is reduced. Based on the results of the kinetic experiments, we propose that the following reactions are predominant,

\[
\begin{align*}
\text{ABTS}^+ + \alpha_1m & \rightleftharpoons \text{ABTS}^- \alpha_1m \\
\text{ABTS}^- \alpha_1m & \rightleftharpoons \text{ABTS}^2+ + \alpha_1m^+ \\
\text{ABTS}^- + \alpha_1m^+ & \rightarrow \text{ABTS}-\alpha_1m
\end{align*}
\]
The results demonstrated that 8–9 ABTS radicals were consumed per $\alpha_m$ molecule, but only 5–6 reduced ABTS molecules formed during the first phase of the reaction. This suggests that 2–3 molecules of ABTS were bound to each molecule of $\alpha_m$ (i.e. the amount of ABTS radicals not converted to reduced ABTS). The purple ABTS conjugation products were localized to at least two different tyrosine residues, Tyr-22 and Tyr-132, supporting the possibility that several ABTS residues can be covalently linked to the same $\alpha_m$ molecule. It is also possible that ABTS conjugation products may be linked to additional locations besides Tyr-22 and Tyr-132, because not all of the purple Pronase and trypsin ABTS-$\alpha_m$ digestion products could be identified.

Based on the products formed in the analogous reactions of ABTS radical with $p$-hydroxybenzoic acid (41) and the plant flavonoid naringin (46), we propose that a tyrosyl radical on $\alpha_m$ reacts with the ABTS radical via the reaction shown in Fig. 12B. The product formed in this reaction has the required molecular mass of 451 $m/z$ for the product identified when the purple $\alpha_m$ protein was digested with Pronase.

We propose a tentative reaction scenario for radical scavenging by $\alpha_m$ (see Fig. 12A) based on the above reactions and the recent finding that $\alpha_m$ has catalytic reductase properties, involving the unpaired thiol group of Cys-34 in the reactive center (8). However, our experiments with the alkylation of the Cys-34 thiol plus its mutation to a serine residue demonstrated that Cys-34 was not solely responsible for reduction of ABTS radicals. This result invokes two possible explanations for the reductant activity of $\alpha_m$. Either an unidentified residue reduces ABTS radicals and its reductant activity is optimized by Cys-34, or an unidentified residue and Cys-34 both reduce ABTS radicals. Reaction of the cysteine thiol is expected to be favorable, because cysteine reduces the ABTS radical with a second order rate constant of $1.9 \times 10^6$ $M^{-1} s^{-1}$ (47). The value of $k_{app}$ for reduction of ABTS radicals by $\alpha_m$ that we obtained was 300-fold less than this rate constant. However, this is expected, because $k_{app}$ will be a function of the rate constants for the reversible reaction between ABTS radical and the thiol group plus that for binding of ABTS radical to $\alpha_m$.

Once the incipient radicals are formed on the $\alpha_m$, they must become localized to Cys-34, which in turn oxidizes tyrosine residues to tyrosyl radicals. These tyrosyl radicals would then covalently couple with the ABTS radical to form the purple adduct (Fig. 12). This proposal is supported by our finding that the purple adduct was formed only when the Cys-34 thiol group was present in the protein. Further-
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more, cysteine thiol radicals are capable of one-electron oxidation of tyrosine residues (48). The reaction of the thiol radical with tyrosine residues need not be direct, because tyrosine residues can be the ultimate sink for oxidizing equivalents in proteins (49), which reflects the thermodynamic pecking order of free radicals (50). Reduction of the thiol radicals by tyrosine residues is a repair reaction that enables superstoichiometric scavinging of ABTS radicals. Thus, we have compelling evidence that when radicals are formed on $\alpha_1$m, they are transferred through the protein and localized to tyrosyl residues. Analogous radical exchange reactions between tyrosine and cysteine residues account for the catalytic activity of ribonucleotide reductases (51).

According to models of the three-dimensional structure of $\alpha_1$m, Cys-34 is located on a large $\omega$-1 flexible loop (22). This would make it accessible to oxidants that bind to $\alpha_1$m. Furthermore, it is likely that the thiol interacts with adjacent lysyl residues, because it was recently shown that the catalytic reductase properties of $\alpha_1$m are dependent on Cys-34 as well as the lysyl residues, Lys-92, Lys-118, and K130A (8). The positively charged lysyl residues may form ionic interactions with the thiolate and consequently lower its $pK_a$. This would facilitate the ability of the Cys-34 thiol to be oxidized and reduce compounds, such as ABTS radicals (Fig. 12).

It has been known for more than 30 years that $\alpha_1$m is modified by extremely heterogeneous yellow-brown chromophores. These have been studied extensively, and it was reported previously that the Cys-34 side chain (20) and several lysyl side chains (21) of $\alpha_1$m isolated from urine or amniotic fluid (29) were modified. In the first report, the modifications could not be identified, and in the second report the sizes of some of the modifications were determined to be 112, 206, and 282 mass units. In the third report, they were structurally identified as derived from the tryptophan metabolite kynurenine. Furthermore, $\alpha_1$m has been shown to react with hemoglobin and heme (8, 27, 52), and it was hypothesized that the chromophores are degradation products of protoporphyrin (27, 36). In this paper, we have shown that in vitro reaction of $\alpha_1$m with ABTS radical yields purple modifications on at least two tyrosyl residues, and these could be identified as fragments of ABTS.

Thus, a picture emerges of the lipocalin reacting with various organic radicals by reduction and covalent addition to several of its side chains. A potential physiological function of $\alpha_1$m could be for it to act as a “radical sink” via its radical reductase and scavenging activities. $\alpha_1$m is found in all extracellular fluids in levels similar to the plasma concentration (i.e. around 2 $\mu$M) (53), which we have shown to display significant radical reductase and scavenging activity. In support of this proposal, we found that $\alpha_1$m was able to prevent an increase in fluorescence of oxidation products of the dipeptide Gly-Tyr. This and related peptides are oxidized by peroxidases to radical species (45). Tyrosyl radicals and oxidation products of tyrosine, such as dopa, are known to promote oxidation in biological systems (54, 55). The most plausible explanation for the action of $\alpha_1$m is that it reduced either tyrosyl radicals or a related oxidant product when Gly-Tyr was oxidized by laccoperidoxase. We are currently investigating the mechanisms by which $\alpha_1$m prevents the fluorescence changes associated with oxidation of Gly-Tyr, and the objects of future studies should be to identify as many as possible of its targets in normal and pathological conditions and to characterize the reaction mechanisms in detail.

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