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N-acetyl-L-methionine is a superior protectant of human serum albumin against photo-oxidation and reactive oxygen species compared to N-acetyl-L-tryptophan

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ABSTRACT

Background: Sodium octanoate (Oct) and N-acetyl-L-tryptophan (N-AcTrp) are widely used as stabilizers during pasteurization and storage of albumin products. However, exposure to light photo-degrades N-AcTrp with the formation of potentially toxic compounds. Therefore, we have examined the usefulness of N-acetyl-L-methionine (N-AcMet) in comparison with N-AcTrp for long-term stability, including photo stability, of albumin products.
Methods: Recombinant human serum albumin (rHSA) with and without additives was photo-irradiated for 4 weeks. The capability of the different stabilizers to scavenge reactive oxygen species (ROS) was examined by ESR spectrometry. Carbonyl contents were assessed by a spectrophotometric method using fluoresceinamine and Western blotting, whereas the structure of rHSA was examined by SDS-PAGE, far-UV circular dichroism and differential scanning calorimetry. Binding was determined by ultrafiltration.
Results: N-AcMet was found to be a superior ROS scavenger both before and after photo-irradiation. The number of carbonyl groups formed was lowest in the presence of N-AcMet. According to SDS-PAGE, N-AcMet stabilizes the monomeric form of rHSA, whereas N-AcTrp induces degradation of rHSA during photo-irradiation. The decrease in α-helical content of rHSA was the smallest in the presence of Oct, without or with N-AcMet. Photo-irradiation did not affect the denaturation temperature or calorimetric enthalpy of rHSA, when N-AcMet was present.
Conclusion: The weakly bound N-AcMet is a superior protectant of albumin, because it is a better ROS-protector and structural stabilizer than N-AcTrp, and it is probable and also useful for other protein preparations.
General significance: N-AcMet is an effective stabilizer of albumin during photo-irradiation, while N-Ac-Trp promotes photo-oxidative damage to albumin.

1. Introduction

Human serum albumin (HSA) is the most abundant protein in plasma, and, in addition to being the primary colloid, it serves as an important transport and depot protein [1,2]. Large amounts of albumin are used clinically during surgery and to treat shock trauma. As, at present, the only source of HSA for clinical application is donated human blood, the risk of transmitting pathogenic viruses, such as those causing hepatitis, HIV, and as yet unidentified diseases, exists. Pasteurization of HSA is carried out by heating at 60 °C for several hours with sodium octanoate (Oct) and N-acetyl-L-tryptophanate (N-AcTrp) as commonly used stabilizers [3], a process that usually destroys the viruses present. These commonly used additives effectively protect HSA by increasing the melting temperature as determined by differential scanning calorimetry (DSC) and decreasing the formation of aggregates after heating [4]. We have previously shown that Oct has the greatest stabilizing effect against heat, while N-AcTrp diminishes oxidation of HSA during pasteurization [5]. However, during storage of the above mentioned albumin products, and most probably also of recombinant HSA (rHSA) and albumin fusion proteins, the presence of N-AcTrp is problematic, because it has low photo-stability which could result in the formation of metabolites giving rise to adverse clinical effects [6–8]. In fact,

Abbreviations: HSA, human serum albumin; Oct, octanoate; N-AcMet, N-acetyl-L-methionine; N-AcTrp, N-acetyl-L-tryptophan; DSC, differential scanning calorimetry; CD, circular dichroism

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L-tryptophan can be easily oxidized following light exposure, leading to significant photo-degradation, via the generation of reactive oxygen species (ROS). Thus, to provide stable and risk-free albumin products, which can withstand prolonged storage and exposure to light, it is necessary to find one or more new additives which can replace N-AcTrp.

All amino acids and amino acid residues of proteins are susceptible to oxidative modification by one or more forms of ROS [9]. Among these, the sulfur-containing amino acids, L-cysteine (Cys) and L-methionine (Met), are easily oxidized. In particular, Met is susceptible to oxidation by almost all forms of ROS [10]. In accordance with these observations, we have previously found that modified Met, i.e. N-acetyl-L-methionine (N-AcMet), is as effective as N-AcTrp in stabilizing albumin during the pasteurization process [10]. However, until now we had not examined the usefulness of N-AcMet for long-term stability, including photo stability, of albumin products.

In the present study, we have exposed rHSA without and with N-AcMet, N-AcTrp and/or Oct to photo-oxidation for 4 weeks. Any oxidation of the protein was examined by direct measurement of carbonyl content by a spectrophotometric method, SDS-PAGE and Western blot analysis, and any effects on the structure were studied by far-UV circular dichroism (CD) and DSC. Because our photo-oxidation system mainly produces hydroxyl radicals, we have also investigated the capability of the different rHSA preparations to scavenge this type of ROS. The results revealed that N-AcMet has a superior stabilizing and protective effect on rHSA. In addition, the scavenging activity of N-AcMet itself is better than that of N-AcTrp, which actually promotes photo-oxidative damage of albumin. Thus, we can propose N-AcMet as a new and safer stabilizer for albumin solutions. Ultrafiltration studies showed that N-AcMet binds to rHSA with only a low affinity. Therefore, N-AcMet should also be useful as a stabilizer for storage of other solubilized proteins.

2. Materials and methods

2.1. Materials

rHSA was donated by Nipro Corporation (Shiga, Japan) and defatted using charcoal treatment as described by Chen [11]. After dialysis against distilled water, the protein was freeze-dried and stored at −20 °C until use. The additive N-AcTrp was purchased from Wako (Tokyo, Japan), whereas N-AcMet and Oct were bought from MP Biomedicals (Solon, OH, USA). [Methyl-^{3}H]-N-AcMet was from Moravek Biochemicals (Brea, CA, USA); the specific activity was 36.0 Ci/mmol, and the radiochemical purity was 98.8%. All solutions were prepared in deionized and distilled water and kept in a sterile room.

2.2. Hydroxyl radical (•OH) scavenging activity of additives

Hydroxyl radicals (•OH) were generated by iron-catalyzed Haber–Weiss reaction (Fenton driven Haber–Weiss reaction), and the generated hydroxyl radicals rapidly reacted with the nitrone spin trap DMPO [12]. The resultant DMPO-OH adduct was detectable with an ESR spectrometer. In practice, additives (0.2 ml) with various concentrations were mixed with DMPO (0.3 M, 0.2 ml), Fe_2SO_4 (0.5 mM, 0.2 ml) and H_2O_2 (4 mM, 0.2 ml) in a sodium phosphate buffer solution (pH 7.4), and then transferred into 100 μl quartz capillary tubes. After 2 min, the ESR spectrum was recorded using an X-band ESR spectrometer (JES-FA100, JEOL Ltd., Tokyo, Japan) under the following conditions: microwave frequency 9.417 GHz, microwave power 8 mW, field modulation 0.1 mT at 100 kHz, and sweep time 2 min. After recording the EPR spectra, the signal intensities of the DMPO-OH adducts were normalized against that of a manganese oxide (Mn^{2+}) signal, where Mn^{2+} is an internal control. The scavenging activity was calculated from the relative intensity peak height of the DMPO-OH EPR signal [13].

2.3. Radical scavenging activity of rHSA in the presence and absence of additives before and after photo-irradiation

rHSA with and without additives were photo-irradiated using photo-stability testing at 40 °C for 4 weeks. During photo-irradiation, the rHSA concentration was 20 μM, and that of any varied additive; the medium was 67 mM sodium phosphate buffer, pH 7.4. The solutions were placed in a light-irradiation tester, PTH-400NC (Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan), equipped with a D65 fluorescent lamp. An infrared cutoff filter of 260–400 nm, with a maximum at 370 nm, was used, and the illuminance was set at 7000 lx. After photo-irradiation for 4 weeks, the hydroxyl radical scavenging activity of the different rHSA preparations was measured by the ESR method described in Section 2.2. For comparison, the hydroxyl radical scavenging activity of identical rHSA solutions which had not been photo-irradiated was also determined.

2.4. The level of oxidized rHSA in the presence and absence of additives after photo-irradiation

rHSA with and without additives were photo-irradiated using photo-stability testing as mentioned above. The different preparations were electrophoresed on duplicate SDS-PAGE gels [14], and the levels of oxidized rHSA were estimated by measuring carbonyl contents and by using Western blotting. Protein-bound carbonyl groups were quantitated using the method of Climent et al. [15]. In short, the groups were derivatized with fluoresceinamine, and their number calculated from the absorbance of the complexes at 490 nm (Jasco Ubest-35 UV/VIS spectrophotometer). The level of oxidized rHSA was also measured by Western blot analysis as previously described by Anraku et al. [16]. Samples were derivatized with dinitrophenylhydrazine (DNP) using an OxyBlot Kit (Serologicals Corporation, Norcross, GA, USA). DNP and protein blots were scanned using the same size section of the blot for each scan as previously described by Anraku et al. [16].

2.5. The structural stability of rHSA in the presence and absence of additives

Far-UV CD spectra (200–250 nm) were recorded with a Jasco J-720 spectropolarimeter (Tokyo, Japan) using a 1-mm path length cell. The concentration of rHSA was 10 μM and that of each additive was 50 μM; the medium was 67 mM sodium phosphate buffer, pH 7.4 and 25 °C. The following six samples were prepared: (1) rHSA alone, (2) rHSA with Oct, (3) rHSA with N-AcTrp, (4) rHSA with N-AcMet, (5) rHSA with N-AcTrp and Oct, and (6) rHSA with N-AcMet and Oct. CD spectra of the six samples were recorded before photo-irradiation and after photo-irradiation to 40 °C for 4 weeks.

Differential scanning calorimetry was carried out after photo-irradiation and by using a Nano-DSC (TA Instruments, Newcastle, USA) using heating rates of 1 K/min. The protein concentration was 20 μM and that of any additive was 100 μM in 67 mM sodium phosphate buffer, pH 7.4. The calorimetric reversibility of the thermally induced transition was checked by reheating the protein solutions in the calorimetric cell, flushed with nitrogen, after cooling from the first run. The results showed, as also observed by Picó [17], that heating to or above 85 °C caused irreversible denaturation. The data obtained from DSC were applied to nonlinear fitting algorithms to calculate the thermodynamic parameters, thermal denaturation temperature (T_m), calorimetric enthalpy (ΔH_{cal}) and van't Hoff enthalpy (ΔH_v), from the temperature dependence of excess molar heat capacity, C_p, by employing using Origin™ scientific plotting software.

2.6. Binding experiments

Binding of N-AcMet (1–100 μM) to rHSA (40 μM) was determined by ultrafiltration using 67 mM sodium phosphate, pH 7.4 and 25 °C, as the buffer. Ultrafiltration was performed using 0.9 ml-samples and an

Amicon MPS-1 micropartition system with YMT ultrafiltration membranes (2000 ×*g*, 40 min). Ligand concentrations in the ultrafiltrate, representing free ligand concentrations (C_f), were determined by liquid-scintillation counting in a LSC-5000 from Aloka (Tokyo, Japan).

For obtaining the relation between N-AcMet concentration and radioactive counting, the radioactivity of the N-AcMet-containing samples, with known total ligand concentration (C_t), was determined before ultrafiltration. Binding parameters were determined by fitting the experimental data to the following Scatchard equation using a non-linear squares program (MULTI program).

$$r = \frac{Cb}{Pt} = \sum_{i=1}^{m} \frac{niKiCf}{1 + KiCf}. \quad (1)$$

In this equation, r is the average number of ligand molecules bound per molecule of protein, n_i is the number of binding sites and K_i is the corresponding association constant in the ith binding class. P_t is the concentration of total protein, and the concentration of bound ligand (C_b) was calculated as $C_t - C_f$.

2.7. Statistics

Statistical significance was evaluated using ANOVA followed by the Newman–Keuls method for comparisons of more than two means. A value of $P < 0.05$ was regarded as statistically significant. Results are reported as mean ± S.D.

3. Results

3.1. ˙OH scavenging activity of additives

First, the ˙OH scavenging activity of N-AcMet and N-AcTrp was examined using ESR spectrometry. As seen in Fig. 1, the scavenging activity of both additives increased in a dose-dependent manner. Furthermore, the ˙OH scavenging activity of N-AcMet was significantly higher than that of N-AcTrp.

3.2. The scavenging activities of rHSA in the presence and absence of additives before and after photo-irradiation

Next, the ˙OH scavenging activity of rHSA with additives before and after photo-irradiation was examined. Before photo-irradiation, rHSA alone or with Oct has only a minor scavenging activity. By contrast, the presence of N-AcTrp and, especially, N-AcMet results in much more pronounced and dose-dependent activities. These activities were not improved by also adding Oct, on the contrary they were slightly diminished (Fig. 2A). Also after photo-irradiation, the ˙OH scavenging activity of N-AcMet was dose-dependent and superior to that of N-AcTrp. In this situation, the effect of Oct alone was hardly detectable, and again no improving effect of Oct on the activities of N-AcMet and N-AcTrp was observed (Fig. 2B). Thus, whether irradiated or not N-AcMet is a better scavenger than N-AcTrp.

3.3. The level of oxidized rHSA after photo-irradiation in the presence and absence of additives

3.3.1. Carbonyl contents

The extent of oxidation of rHSA after photo-irradiation was evaluated by measuring the content of carbonyl groups by spectrophotometry. As seen in Fig. 3A, Oct did not affect the number of carbonyl groups formed. By contrast, the presence of N-AcMet or N-AcTrp resulted in a slightly decreased and a pronounced increased oxidation of rHSA, respectively. Oct had no influence on the latter effects.

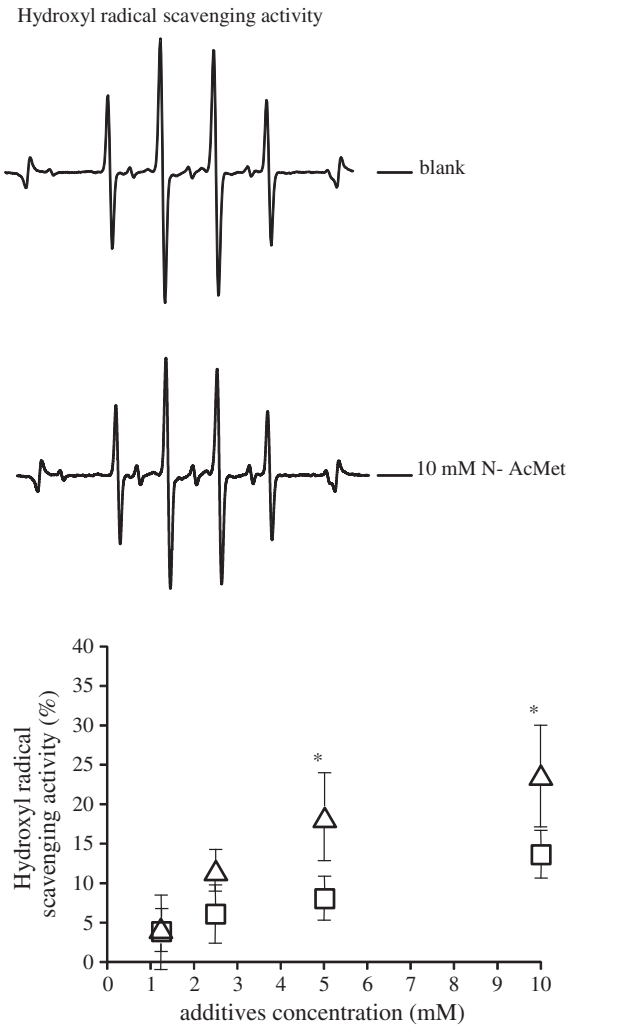

Fig. 1. Relative hydroxyl radical scavenging activities of different concentrations of N-AcMet (△) and N-AcTrp (□). The upper part shows representative spectra without and with N-AcMet. Each point in the lower part represents the mean ± S.D. (n = 3). * $P < 0.05$, compared with N-AcTrp.

3.3.2. SDS-PAGE

Fig. 3B shows the effect of photo-irradiation on the degradation of rHSA using SDS-PAGE. The presence of N-AcTrp alone or together with Oct induced formation of albumin degradation. By contrast, the presence of N-AcMet and/or Oct had no detectable effect on degradation.

3.3.3. Western blotting

The carbonyl contents of photo-irradiated rHSA were also determined by Western blot analysis using an anti-DNP antibody (Fig. 3C). Carbonyl formation in oxidized rHSA was calculated as densitometry ratio of DNP area and protein area. This carbonyl/protein ratio was regarded as relative oxidized rHSA ratio (Fig. 3D). The presence of Oct alone had no effect on the carbonyl formation. Interestingly, addition of N-AcMet decreased slightly the carbonyl/protein ratio both when added alone and in combination with Oct. By contrast, when N-AcTrp or both N-AcTrp and Oct were present, the oxidation ratio increased by 1.45 and 1.38 times, respectively.

3.4. The structural stability of rHSA after photo-irradiation in the presence and absence of additives

3.4.1. DSC-studies

The stabilizing effects of the additives on rHSA during photo-irradiation were studied by DSC (Fig. 4). In all cases shown, a single and

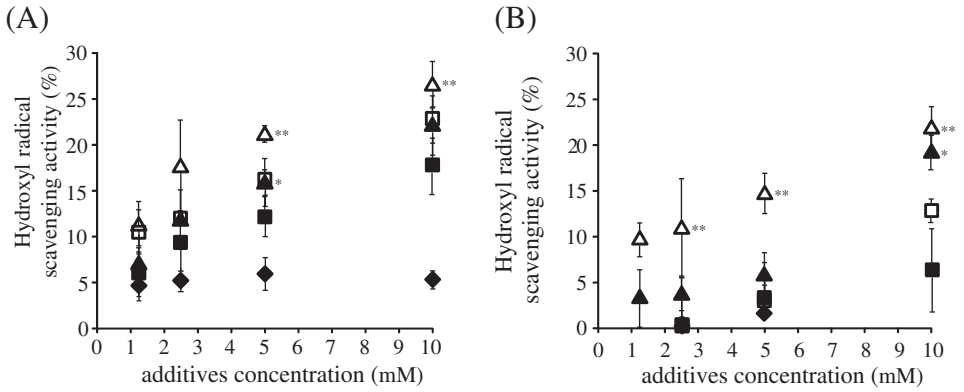

Fig. 2. Relative hydroxyl radical scavenging activity of rHSA in the presence of different concentrations of Oct (♦), N-AcMet (△), N-AcTrp (□), Oct + N-AcMet (▲) and Oct + N-AcTrp (■) before (A) and after (B) photo-irradiation for 4 weeks. The scavenging activity of rHSA alone was 5.5 ± 1.1% before photo-irradiation but very small after photo-irradiation. The concentration of rHSA was 20 μM. Each point represents the mean ± S.D. (n = 3). * $P < 0.05$, compared with rHSA + N-AcTrp + Oct. ** $P < 0.05$, compared with rHSA + N-AcTrp.

sharp endothermic peak was observed of which the size increased in the following order: rHSA without additive < rHSA + N-AcMet < rHSA + Oct + N-AcTrp < rHSA + Oct + N-AcMet < rHSA + Oct. By contrast to these examples, no normal thermogram was obtained after photo-irradiation in the presence of only N-AcTrp. The form of the endotherms in Fig. 4 indicates that thermal denaturation can be explained by a single

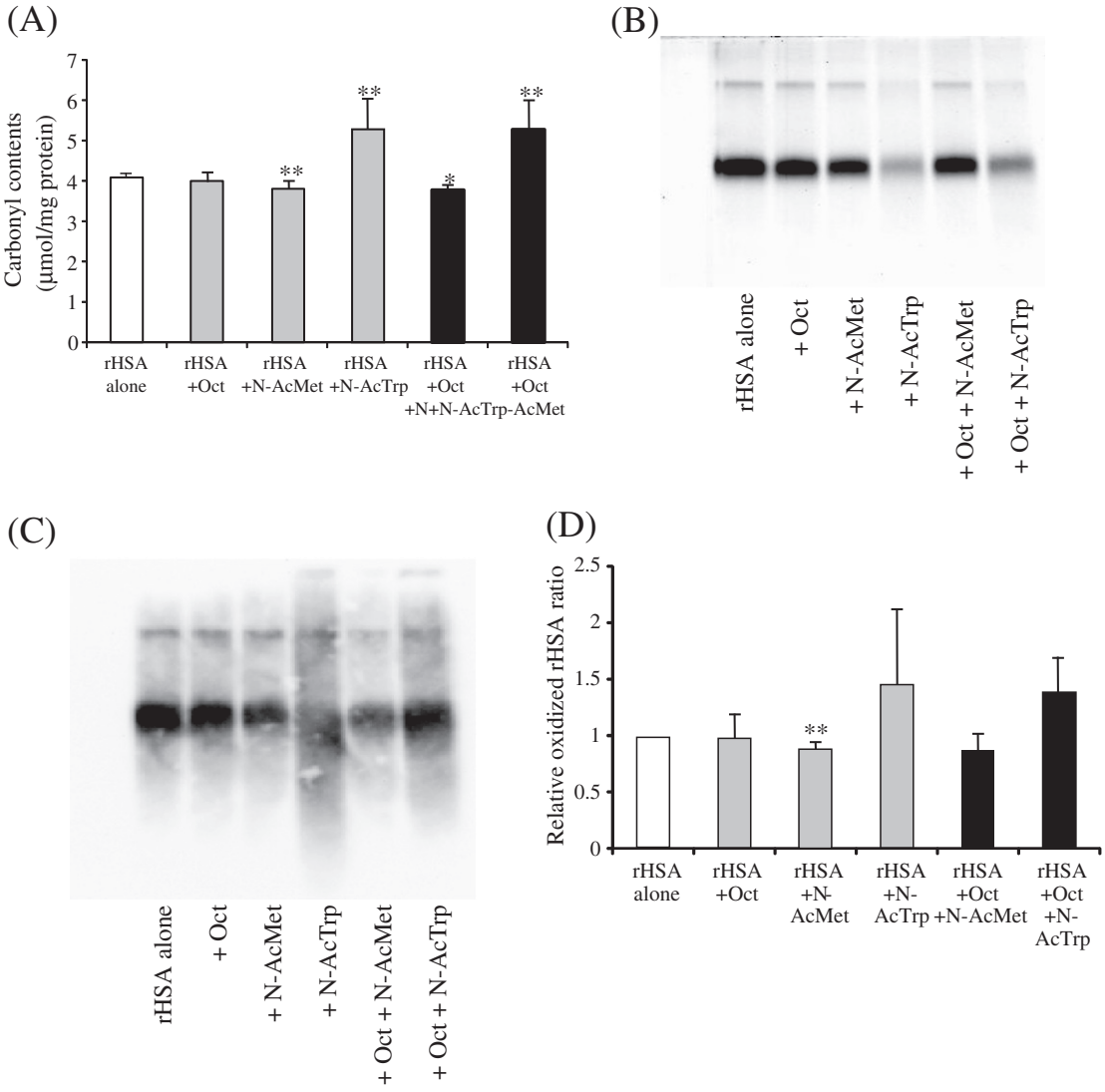

Fig. 3. Evaluation of oxidation of rHSA which had been photo-irradiated in the absence (control) or in the presence of additives. (A) Carbonyl contents of the rHSA preparations. The protein concentration was 20 μM and that of each additive was 100 μM. (B) SDS-PAGE electrophoresis. The concentration of rHSA was 20 μM and that of each additive was 100 μM. (C) Western blots and staining with Oxyblot Kit reagents. (D) Carbonyl formation of oxidized rHSA was determined as densitometry ratio of DNP area and protein area. Each column in (A) and (D) represents the mean ± S.D. (n = 3–4). * $P < 0.05$, ** $P < 0.01$, compared with rHSA in the absence of additives.

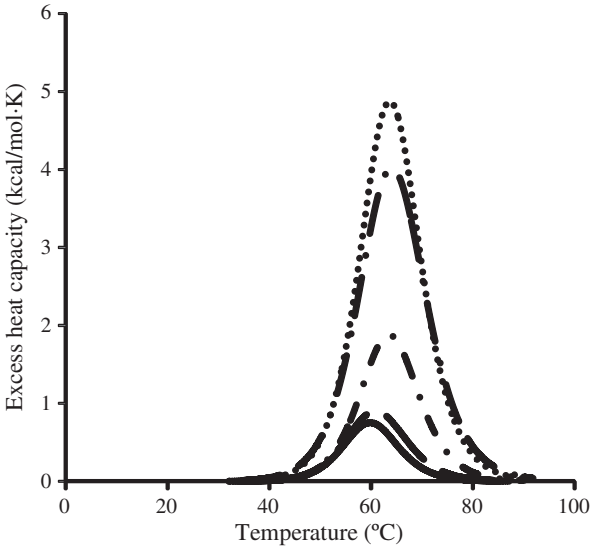

Fig. 4. Effects of additives on the thermogram of rHSA obtained by DSC after photo-irradiation for 4 weeks. Results are shown for rHSA alone (——), rHSA + Oct (·····), rHSA + N-AcMet (— —), rHSA + Oct + N-AcMet (— · —) and rHSA + Oct + N-AcTrp (– · –). The concentration of rHSA was 20 μM and that of each additive was 100 μM.

Table 1
Thermodynamic data obtained from DSC of different rHSA samples after photo-irradiation.[a]

Protein samples	T_m (°C)	ΔH_{cal} (kcal/mol)	$\Delta H_v/\Delta H_{cal}$
rHSA alone	59.1 ± 0.05	10.1 ± 1.0	6.0 ± 0.10
rHSA + Oct	64.3 ± 0.09	73.7 ± 1.5	0.2 ± 0.05
rHSA + N-AcMet	59.7 ± 0.07	13.6 ± 4.9	4.2 ± 0.08
rHSA + N-AcTrp	ND[b]	ND	ND
rHSA + Oct + N-AcMet	65.0 ± 0.10	67.4 ± 1.2	0.8 ± 0.05
rHSA + Oct + N-AcTrp	65.0 ± 0.11	27.0 ± 1.1	2.2 ± 0.09

[a] The concentration of rHSA was 20 μM and that of each additive was 100 μM. The results are means ± S.D. (n = 3).
[b] No normal thermogram could be detected.

component model [17,18]. Therefore, single values for T_m, ΔH_{cal} and ΔH_v can be calculated. As apparent from Table 1, also for these parameters in addition of N-AcMet alone has the smallest effect. Thus, N-AcMet seems to be the most effective additive in protecting rHSA during photo-irradiation.

3.4.2. CD spectra

Fig. 5 shows the effect of the additives on the structure of rHSA as determined by circular dichroism. Before photo-irradiation, binding of the additives, individually or in different combinations, did not significantly affect the far-UV CD spectrum of rHSA (Fig. 5A). However, photo-irradiation of the different protein-additive combinations resulted in much less [θ]-values (Fig. 5B). On the basis of the CD spectra we have estimated the α-helical content of the different rHSA preparations (Table 2). As seen, photo-irradiation decreased in all examples the α-helical content of rHSA. The decrease was the smallest in the presence of Oct, with or without N-AcMet, namely 14.2%. In the other cases, the decreases were 21.5–23.9%.

3.5. Binding of N-AcMet to rHSA

A Scatchard analysis of the ultrafiltration data (Fig. 6) proposes the existence of one primary binding site for N-AcMet; $n_1 = 1.14 \pm 0.02$ (n = 4). The corresponding association constant is $5.5 \pm 0.22 \times 10^4\ M^{-1}$. In comparison, the other additives bind with a higher affinity. Thus, both Oct and N-AcTrp bind to one high-affinity site but the K_1-values are $1.3 \times 10^6\ M^{-1}$ and $9.1 \times 10^4\ M^{-1}$, respectively [5].

4. Discussion

The annual production of purified HSA for clinical purposes now exceeds 300 t [1,2]. In addition, rHSA and albumin fusion proteins are soon ready for clinical use. Therefore, it is surprising that only sparse information can be found about the protective effects of the standard additives Oct and N-AcTrp or about their potential adverse effects during storage and exposure to light. In the absence of sensitizing compounds, the occurrence of any photo-toxicity is dependent on the direct absorption of the appropriate wavelength of sunlight, especially UVA (320–400 nm) and UVB (290–320 nm). In the case of albumin solutions, it is of great relevance to note that N-AcTrp can be easily oxidized by light exposure, and thereby photo-degraded via the generation of ROS, and some of these oxidized products could have adverse effects. In a separate study, we observed that N-AcMet is as efficient as N-AcTrp in protecting HSA against two oxidizing chemicals, i.e., AAPH and DPPH [10]. Therefore, in the present study we investigated the protective effects of N-AcMet against ROS and photo-oxidation during prolonged storage, because if this compound is as efficient as N-AcTrp in protecting HSA, or even better, the risk of toxic, oxidized products could be minimized.

4.1. The antioxidant potential of the different additives

The hydroxyl radical and other reactive oxygen species generated by light exposure can cause damage to solubilized protein in a direct way

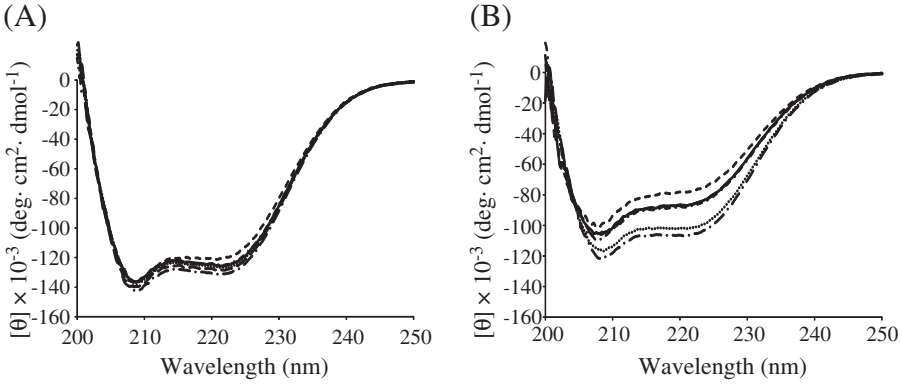

Fig. 5. Effects of additives on the far-UV CD spectrum of rHSA. (A) Before photo-irradiation. (B) After photo-irradiation for 4 weeks. Results are shown for rHSA alone (——), rHSA + Oct (·····), rHSA + N-AcMet (— —), rHSA + N-AcTrp (– – –), rHSA + Oct + N-AcMet (— · —) and rHSA + Oct + N-AcTrp (– · –). The concentration of rHSA was 10 μM and that of each additive was 50 μM.

Table 2
Alpha helical content of different rHSA preparations calculated from CD spectra before and after photo-irradiation.[a]

Protein samples	rHSA alone	rHSA + Oct	rHSA + N-AcMet	rHSA + N-AcTrp	rHSA + Oct + N-AcMet	rHSA + Oct + N-AcTrp
(A) Before photo-irradiation (%)	78.5 ± 1.9	79.1 ± 2.0	80.2 ± 1.8	75.3 ± 2.1	81.6 ± 1.5	78.2 ± 2.4
(B) After photo-irradiation (%)	56.2 ± 2.4	64.8 ± 2.0	56.3 ± 1.9	51.6 ± 1.9	67.4 ± 1.6	56.7 ± 1.8
(A)–(B) (%)	22.3 ± 1.2	14.2 ± 1.3	23.9 ± 2.3	23.8 ± 2.1	14.2 ± 1.1	21.5 ± 2.2

[a] The concentration of rHSA was 10 μM and that of each additive was 50 μM. The results are means ± S.D. (n = 3).

[19]. We found that N-AcMet is superior to N-AcTrp in scavenging $^{\bullet}$OH and to protect rHSA both against $^{\bullet}$OH and prolonged exposure to light. By contrast, Oct had no or only a small scavenging effect. The pronounced scavenging activity, and thereby protective effect, of N-AcMet is not the result of strong binding to rHSA. By contrast, the binding constant for N-AcMet is lower than that for binding of N-AcTrp; namely 5.5×10^4 M^{-1} versus 9.1×10^4 M^{-1}. That of Oct is even higher: 1.3×10^6 M^{-1}. From data on the rate constants of reactions of the $^{\bullet}$OH with rHSA and N-AcMet, it can be calculated that 98% of any $^{\bullet}$OH radicals generated would react with the amino acid, not the protein. Thus, due to its pronounced scavenging efficiency, and because strong protein binding is not a prerequisite, N-AcMet should also be useful as a stabilizer and protector not only for storage of different albumins but also for storage of other solubilized proteins.

4.2. The structural stability of rHSA with additives after photo-irradiation

Any stabilizing effect of the additives on the structure of rHSA during photo-irradiation was evaluated by DSC and CD dichroism. The denaturation temperature, T_m, for rHSA was almost the same, whether N-AcMet was present or not during photo-irradiation. By contrast, T_m was increased by 5–6 °C, when photo-irradiation took place in the presence of Oct alone or together with N-AcMet or N-AcTrp. ΔH_{cal} is an index of the transition process to the denaturation states of proteins during thermal denaturation [18], and it is generally thought to reflect the hydration of hydrophobic regions buried in the native protein structure during the unfolding process. In general, the changes in the ΔH_{cal}-values varied in parallel with the changes in the T_m-values (Table 1). However, in contrast to N-AcMet the presence of N-AcTrp greatly diminished the protection of Oct during photo-irradiation. ΔH_{cal} and ΔH_v represents calorimetric enthalpy and van't Hoff enthalpy, respectively, and the ratio between these values is also an index of the transition process to the denaturation states of proteins during thermal denaturation [20]. The value calculated for the photo-irradiated sample having both Oct and N-AcMet is almost the same as that calculated for the untreated sample, whereas those calculated for the samples with Oct alone or together with N-AcMet or N-AcTrp are decreased. Taken together, the results of Table 1 propose a stabilizing effect of Oct on the structure of rHSA during photo-irradiation. This effect is largely unaffected by the simultaneous presence of N-AcMet, whereas that of N-AcTrp decreases stability. This conclusion is supported by the finding that irradiation of rHSA in the presence of N-AcTrp alone results in a protein product, which has no normal thermogram. The denaturing effect was also evident from a SDS-PAGE analysis (Fig. 3B), and it could be due to N-AcTrp-induced photo-degradation of rHSA.

The stabilizing effect of Oct is also superior to those of N-AcMet and N-AcTrp when determining the α-helical content of rHSA before and after photo-irradiation (Table 2). In contrast to N-AcMet, N-AcTrp abolishes the positive effect of Oct when the two compounds are added together. Thus, also when using this parameter, N-AcTrp is not able to protect the structure of rHSA during photo-irradiation.

Normally, photo-stability testing is carried out under the ICH Harmonized Tripartite Guideline. For confirmatory studies, samples should be exposed to light providing an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 W hours/square meter to allow direct comparisons to be made between the drug substance and drug product. In our study, rHSA with or without additives were exposed to light providing an overall illumination of more than 1.2 million lux hours. Thus, it may be necessary for the formulation of albumin products to be reassessed in the light of photo-stability.

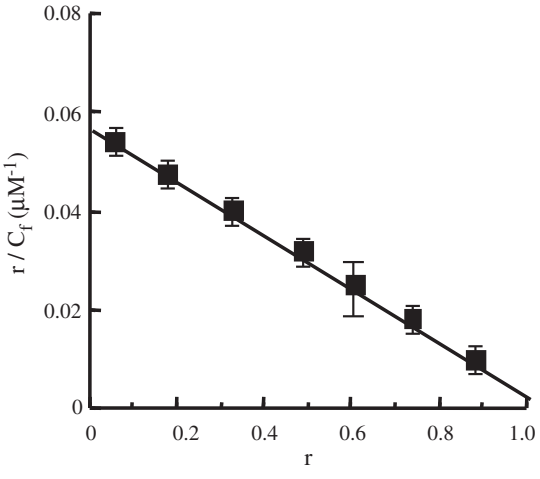

Fig. 6. Scatchard plot of the binding of N-AcMet to rHSA as determined by ultrafiltration. Each point represents the mean ± S.D. (n = 4).

5. Conclusion

Apparently, the roles of Oct, N-AcTrp and N-AcMet for the photo-stability of albumin have not been studied before. We found that N-AcMet is superior to N-AcTrp with respect to scavenge ROS and to protect the protein against oxidation. Furthermore, N-AcMet, with or without Oct, has a good stabilizing effect on the structure of albumin. By contrast, N-AcTrp promotes photo-oxidative degradation of the protein. Thus, N-AcMet should be useful as a new stabilizer and antioxidant for albumin preparations.

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References

[1] T. Peters Jr., All about Albumin: Biochemistry, Genetics, and Medical Applications, Academic Press, San Diego, California, 1996.
[2] M. Otagiri, V.T. Chuang, T. Maruyama, U. Kragh-Hansen, Human Serum Albumin, Sojo University Publishing Center, Kumamoto, Japan, 2013.
[3] A. Shrake, J.S. Finlayson, P.D. Ross, Thermal stability of human albumin measured by differential scanning calorimetry. I. Effects of caprylate and N-acetyltryptophanate, Vox Sang. 47 (1984) 7–18.
[4] T. Arakawa, Y. Kita, Stabilizing effects of caprylate and acetyltryptophanate on heat-induced aggregation of bovine serum albumin, Biochim. Biophys. Acta 1479 (2000) 32–36.
[5] M. Anraku, Y. Tsurusaki, H. Watanabe, T. Maruyama, U. Kragh-Hansen, M. Otagiri, Stabilizing mechanisms in commercial albumin preparations: octanoate and N-acetyl-L-tryptophanate protect human serum albumin against heat and oxidative stress, Biochim. Biophys. Acta 1702 (2004) 9–17.

[6] A. Aguilera, R. Selgas, J.J. Diez, M.A. Bajo, R. Codoceo, V. Alvarez, Anorexia in end-stage renal disease: pathophysiology and treatment, Expert Opin. Pharmacother. 2 (2001) 1825–1838.
[7] N. Igarashi, S. Onoue, Y. Tsuda, Photoreactivity of amino acids: tryptophan-induced photochemical events via reactive oxygen species generation, Anal. Sci. 23 (2007) 943–948.
[8] J. Topczewska-Bruns, D. Pawlak, A. Tankiewicz, E. Chabielska, W. Buczko, Kynurenine metabolism in central nervous system in experimental chronic renal failure, Adv. Exp. Med. Biol. 527 (2003) 177–182.
[9] W. Vogt, Oxidation of methionyl residues in proteins: tools, targets, and reversal, Free Radic. Biol. Med. 18 (1995) 93–105.
[10] M. Anraku, Y. Kouno, T. Kai, Y. Tsurusaki, K. Yamasaki, M. Otagiri, The role of N-acetyl-methioninate as a new stabilizer for albumin products, Int. J. Pharm. 329 (2007) 19–24.
[11] R.F. Chen, Removal of fatty acids from serum albumin by charcoal treatment, J. Biol. Chem. 242 (1967) 173–181.
[12] J.Y. Je, P.J. Park, S.K. Kim, Free radical scavenging properties of hetero-chitooligosaccharides using an ESR spectroscopy, Food Chem. Toxicol. 42 (2004) 381–387.
[13] Y. Iwao, Y. Ishima, J. Yamada, T. Noguchi, U. Kragh-Hansen, K. Mera, D. Honda, A. Suenaga, T. Maruyama, M. Otagiri, Quantitative evaluation of the role of cysteine and methionine residues in the antioxidant activity of human serum albumin using recombinant mutants, IUBMB Life 64 (2012) 450–454.
[14] H. Schagger, G. von Jagow, Tricine–sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, Anal. Biochem. 166 (1989) 368–379.
[15] I. Climent, L. Tsai, R.L. Levine, Derivatization of γ-glutamyl semialdehyde residues in oxidized proteins by fluoresceinamine, Anal. Biochem. 182 (1989) 226–232.
[16] M. Anraku, K. Kitamura, A. Shinohara, M. Adachi, A. Suenga, T. Maruyama, K. Miyanaka, T. Miyoshi, N. Shiraishi, H. Nonoguchi, M. Otagiri, K. Tomita, Intravenous iron administration induces oxidation of serum albumin in hemodialysis patients, Kidney Int. 66 (2004) 841–848.
[17] G.A. Picó, Thermodynamic features of the thermal unfolding of human serum albumin, Int. J. Biol. Macromol. 20 (1997) 63–73.
[18] M. Anraku, K. Yamasaki, T. Maruyama, U. Kragh-Hansen, M. Otagiri, Effect of oxidative stress on the structure and function of human serum albumin, Pharm. Res. 18 (2001) 632–639.
[19] D.I. Pattison, A.S. Rahmanto, M.J. Davies, Photo-oxidation of proteins, Photochem. Photobiol. Sci. 11 (2012) 38–53.
[20] T. Kosa, T. Maruyama, M. Otagiri, Species differences of serum albumins: II. Chemical and thermal stability, Pharm. Res. 15 (1998) 449–454.