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N-acetyl-L-methionine is a superior protectant of human serum albumin against post-translational oxidation as compared to N-acetyl-L-tryptophan

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ABSTRACT

Sodium octanoate and N-acetyl-L-tryptophan (N-AcTrp) are widely used as stabilizers during pasteurization and storage of albumin products. However, as compared with N-AcTrp, N-acetyl-L-methionine (N-AcMet) is superior in protecting albumin exposed to light during storage. Here, we examine, whether N-AcMet also is better than N-AcTrp to protect albumin against oxidation. Recombinant human serum albumin (rHSA) without and with N-AcMet or N-AcTrp was oxidized by using chloramine-T (CT) as a model compound for mimicking oxidative stress. Oxidation of rHSA was examined by determining carbonyl groups and advanced oxidation protein products. Structural changes were studied by native-PAGE, circular dichroism, intrinsic fluorescence and differential scanning calorimetry. The anti-oxidant capacity of CT-treated rHSA was quantified by its ability to scavenge peroxynitrite and the hydroxyl radical. The pharmacokinetics of indocyanine green-labeled albumin preparations was studied in male mice. We found that the number of chemical modifications and the structural changes of rHSA were significantly smaller in the presence of N-AcMet than in the presence of N-AcTrp. The anti-oxidant properties of CT-exposed rHSA were best protected by adding N-AcMet. Finally, N-AcMet is superior in preserving the normal pharmacokinetics of rHSA. Thus, N-AcMet is superior to N-AcTrp in protecting albumin preparations against oxidation. In addition, N-AcMet is probable also useful for protecting other proteins. Therefore, N-AcMet should be useful as a new and effective stabilizer and antioxidant for albumin isolated from blood, rHSA, albumin-fusion proteins and for preparations of rHSA-therapeutic complexes.

1. Introduction

Human serum albumin (HSA) is a major component of the circulating blood, accounting for approximately 60% of plasma protein, and it is the main contributor to the vascular osmotic pressure [1]. Therefore, albumin preparations have long been used as a plasma substitute in clinical practice, such as for the emergency treatment of shock, the restoration of blood volume, the acute management of burns, and in other clinical situations associated with hypoproteinemia [2]. In addition to its osmotic effect, HSA also has various other functions [1]. For instance, HSA shows a high affinity for many endogenous as well as exogenous substances, such as drugs and toxins, and thereby affects their pharmacokinetics and consequently their physiological activity and drug efficacy [3,4]. HSA also plays an important role as a major anti-oxidant in plasma and extracellular compartments, mainly because the cysteine in position 34 (Cys34) of HSA accounts for approximately 70% of all free sulfhydryl (SH) groups in plasma [5]. These findings suggest that HSA has an important role in the maintenance of biological homeostasis.

Abbreviations: HSA, human serum albumin; rHSA, recombinant HSA; N-AcMet, N-acetyl-L-methionine; N-AcTrp, N-acetyl-L-tryptophan; Oct, octanoate; ROS, reactive oxygen species; CT, chloramine-T; DSC, differential scanning calorimetry; CD, circular dichroism

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Because of its multiple functions, treatment of patients with HSA is expected to have several beneficial effects. At present, the only source of HSA for clinical use is human blood. However, blood can contain viruses or other pathogens, which can cause disease in the recipients. That is why HSA preparations are pasteurized before clinical use. However, due to its long circulating half-life of ca. 19 days, HSA is subject to a variety of post-translational modifications in vivo as a result of various stress conditions, such as oxidation and glycation [1,6]. Thus, albumin products prepared from pooled plasma sources can be post-translationally modified [7]. Such modifications often compromise albumin's functions, other than its osmotic pressure maintaining function. In fact, some research groups have reported that HSA is functionally impaired in a disease state, and that this alteration is associated with disease progression [8,9]. Therefore, having albumin preparations in which the HSA functions during storage and upon administration are preserved with minimal additional modifications would be highly desirable.

Several complexes of recombinant HSA (rHSA) and a therapeutic are now available for clinical use. In addition, other such complexes as well as fusion proteins and rHSA itself are in different types of clinical trials. Also in these cases it is important to be able to store the products without risking modifications such as oxidation.

For stabilizing HSA during pasteurization it is common to add the ligands octanoate (Oct) and N-Acetyl-L-tryptophan (N-AcTrp). We have previously studied the effects of these additives during pasteurization and found that Oct has a good stabilizing effect against heat, whereas the presence of N-AcTrp diminishes oxidation of HSA [10]. More recently, we have studied whether N-Acetyl-L-methionine (N-AcMet) could be used as a photo-stabilizer during prolonged storage instead of N-AcTrp. The problem associated with the use of N-AcTrp for that purpose is that it can be easily oxidized by light exposure, and thereby photo-degraded. The products formed can oxidize HSA and have been reported to be neurotoxic [11]. The reason for using N-AcMet in this respect was that methionine itself is susceptible to oxidation by almost all forms of reactive oxygen species (ROS) and thereby could protect HSA. We found that N-AcMet is an effective protector of albumin against photo-oxidation [12]. However, until now we had not examined the usefulness of N-AcMet for protecting HSA or rHSA against other types of oxidation relevant for storage and administration.

In the present study, therefore, we have examined the protective effect of N-AcMet on the oxidation of rHSA and compared it with that of N-AcTrp. Chloramine-T (CT) was used as a model compound for mimicking oxidative stress. The extent of rHSA modifications and the effects on protein structure were studied by several physico-chemical methods. Likewise were the anti-oxidant properties of CT-exposed rHSA, without and with additive, quantified. Finally, the pharmacokinetics of the different albumin preparations was investigated by using mice. The results revealed that N-AcMet has a superior protective effect on rHSA against oxidation. Therefore, N-AcMet should be very useful as a stabilizer against oxidation during storage and upon administration.

2. Materials and methods

2.1. Materials

Male ddY mice were purchased from Kyudo Co., Ltd, Saga, Japan. rHSA was a gift from Nipro Corporation (Shiga, Japan) and defatted using charcoal treatment as described by Chen [13]. After dialysis against distilled water, the protein was freeze-dried and stored at −20 °C until use. N-AcTrp and CT were purchased from Wako (Tokyo, Japan), and N-AcMet was bought from MP Biomedicals (Solon, OH, USA). Peroxynitrite solution was obtained from Chemical Dojin Co. Ltd. (Kumamoto, Japan). All solutions were prepared in deionized and distilled water and kept in a sterile room.

2.2. Oxidation of rHSA by CT without and with additives

For preparing oxidized rHSA by CT-treatment in the absence and in the presence of N-AcMet (75 μM) or N-AcTrp (75 μM), rHSA (15 μM) was incubated for 1 h in phosphate buffer (pH 7.4) at 37 °C in an oxygen-saturated solution containing CT alone (5 mM) (non-additive CT-HSA) or with N-AcMet (CT-HSA with N-AcMet) or N-AcTrp (CT-HSA with N-AcTrp). This molar ratio between additive and protein was chosen, because HSA used in the clinic, pharmaceutical-grade albumin, is pasteurized and delivered with Oct and N-AcTrp in molar ratios to albumin of approximately 5–1. After incubation, the oxidative processes were stopped by cooling and by dialysing the solutions extensively against water. A control was made by incubating albumin dissolved in buffer alone, and in all cases the proteins were freeze-dried after dialysis and stored at −20 °C until use.

2.3. The extent of rHSA oxidation without and with additives

2.3.1. Carbonyl contents of oxidized rHSA

The degree of rHSA oxidation was evaluated by running native-PAGE on Novex Tris-Glycine gels [14], by measuring carbonyl contents and by using Western blotting. Carbonyl groups were quantitated as described by Climent et al. [15]. Briefly, the carbonyl groups were derivatized with fluoresceinamine, and their number was determined from the absorbance of the complexes at 490 nm (Jasco Ubest-35 UV/VIS spectrophotometer). The level of rHSA oxidation was also quantitated by Western blot analysis as described by Anraku et al. [16]. The protein was derivatized with dinitrophenylhydrazine (DNP) using an OxyBlot Kit (Serologicals Corporation, Norcross, GA, USA). Afterwards, samples were electrophoresed on duplicate SDS-PAGE gels. Following electrotransfer to a polyvinylidene difluoride membrane, one blot was stained for DNP using the OxyBlot Kit reagents. The second blot was stained with Coomassie brilliant blue G for protein. All rHSAs had identical reactivity with polyclonal antiserum using the ELISA method (data not shown). Bands were visualized with chemiluminescent chemicals and captured on film at 10 min DNP and protein blots were scanned using the same size section of the blot for each scan. The results obtained are reported as DNP area/protein area and are given in densitometry units.

2.3.2. Advanced oxidation protein products (AOPP) contents of oxidized rHSA

Protein samples were treated with potassium iodide as described by Witko-Sarsat et al. [17], and AOPP contents were assessed by measurement of the absorbance at 340 nm. Specifically, CT standard solutions 0–100 μmol/L) were placed in the wells of a 96-well microtiter plate, followed by 20 μL of acetic acid. 10 μL of 1.16 M potassium iodide (KI) was then added, followed by 20 μL of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm in a microplate reader against a blank containing 200 μL of phosphate buffer, 10 μL of KI and 20 μL of acetic acid. The CT absorbance at 340 nm was linear within the range of 0–100 μmol/L. AOPP concentrations were expressed in μmol/L of CT equivalents.

2.3.3. Peroxynitrite scavenging activity of oxidized rHSA

The amount of peroxynitrite was evaluated by the extent of dihydrorhodamine 123 oxidation. 50 μL of gelatin (1.2 mg/mL),

50 μL of dihydrorhodamine 123 (0.1 mM) and 50 μL of native or oxidized rHSA were added to a 96-well plate, and then incubated for 5 min Before incubation, 50 μL aliquots of the peroxynitrite solution (0.28 mM) were added to the above prepared solutions. After the 5 min incubation, the fluorescence of the rhodamine 123 formed was measured at 535 nm after having been excited at 500 nm [18,19]. Peroxynitrite scavenging activity was estimated from the decrease in fluoresence of the rhodamine 123 according to the following equation:

$$\text{Radical scavenging activity (\%)} = 100 - (\text{fluorescence with albu min samples} / \text{fluorescence without albu min samples} \times 100). \quad (1)$$

2.3.4. Hydroxyl radical ($^{\bullet}OH$) scavenging activity of oxidized rHSA

Hydroxyl radicals ($^{\bullet}OH$) were formed by iron-catalyzed Haber-Weiss reaction (Fenton driven Haber-Weiss reaction), and they reacted rapidly with the nitrone spin trap DMPO [20]. The resultant DMPO-OH product was detectable with an ESR spectrometer. In practice, 0.2 mL of the different albumin forms (50 μM) 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 to 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 [21], compared to the intensities observed in the absence of oxidized HSAs, using Eq. (2).

$$\text{ROS scavenging activity (\%)} = 100 - (\text{relative intensity with albu min samples/relative intensity without albu min samples} \times 100). \quad (2)$$

2.4. Structural stability of rHSA oxidized without and with additives

2.4.1. Far-UV circular dichroism (CD) spectra of oxidized HSA

Far-UV CD spectra (200–250 nm) were recorded with a Jasco J-720 type spectropolarimeter (Tokyo, Japan) using 1 mm pathlength cells. The final concentration of native and oxidized 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.

2.4.2. Differential scanning calorimetry (DSC)

Differential scanning calorimetry was carried out with the different albumin forms and by using a Nano-DSC (TA Instruments, Newcastle, USA) using heating rates of 1 K/min. The final protein concentration was 20 μM and that of each additive was 100 μM in 67 mM sodium phosphate buffer, pH 7.4. The results showed, as also observed by Picó [22], 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, namely 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.

2.4.3. Effect on aromatic amino acid residues

Steady-state fluorescence measurements were made using a Jasco FP-770 fluorometer with 1 cm quartz cells and thermostated devices. All studies were performed with a final protein concentration of 2 μM at 25 °C using 5 nm excitation and emission band widths. A fluorescence excitation wavelength of 295 nm was employed for mainly exciting the lone tryptophan residue in position 214. Absorbance spectra (200–400 nm) of the albumins (20 μM) were recorded at 25 °C with 1 cm quartz cells by using the Jasco UV/VIS spectrophotometer.

2.5. In vivo fluorescence imaging of rHSA oxidized without and with additives

All animal experiments were approved by the Animal Care and Use Committee of Sojo University. Care and handling of the animals were performed in accordance with the guidelines, principles, and procedures for the care and use of laboratory animals of Sojo University. All male, 7–9 week-old ddY mice were maintained under conventional housing conditions. The animals were acclimated for 1 week before the experiments. Prior to the experiments, the mice fasted overnight but had free access to water. ICG (Indocyanine green)-labeled rHSA was prepared using ICG Labeling Kit-NH_2 (Dojindo Molecular Technologies, Inc). The mice were injected with 1 mg of native or oxidized rHSA labeled with ICG per kg i.v. At different times after injection, blood was collected from the vena cava under ether anesthesia, and plasma was obtained by centrifugation. The fluorescence of the plasma samples was measured using the Jasco FP-770 fluorometer (excitation at 774 nm and emission at 805 nm) [23].

2.6. Statistics

Pharmacokinetic analyses of the samples were conducted using a non-compartment model. Pharmacokinetic parameters were calculated using the moment analysis program available with Microsoft Excel (Microsoft Corp.). Data are shown as the mean ± S.D. for the indicated number of animals. Other results are also reported as mean ± S.D. 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.

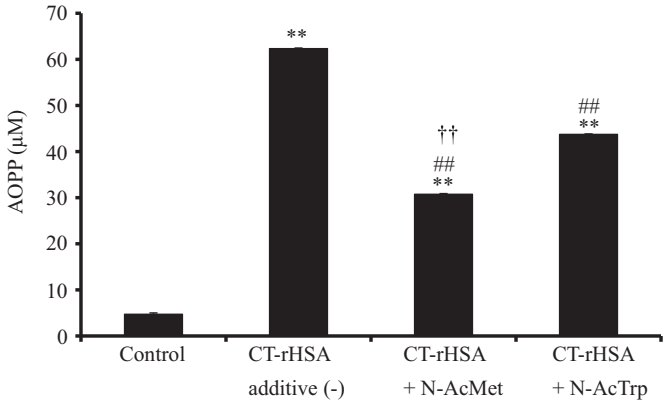

Fig. 1. AOPP content of non-treated rHSA (control) and oxidized rHSAs. The concentration of rHSA was 100 μM. Each column represents the mean ± S.D. (n=4). ** $P < 0.01$, compared with control. ## $P < 0.01$, compared with CT-rHSA additive (-). †† $P < 0.01$, compared with CT-rHSA + N-AcTrp.

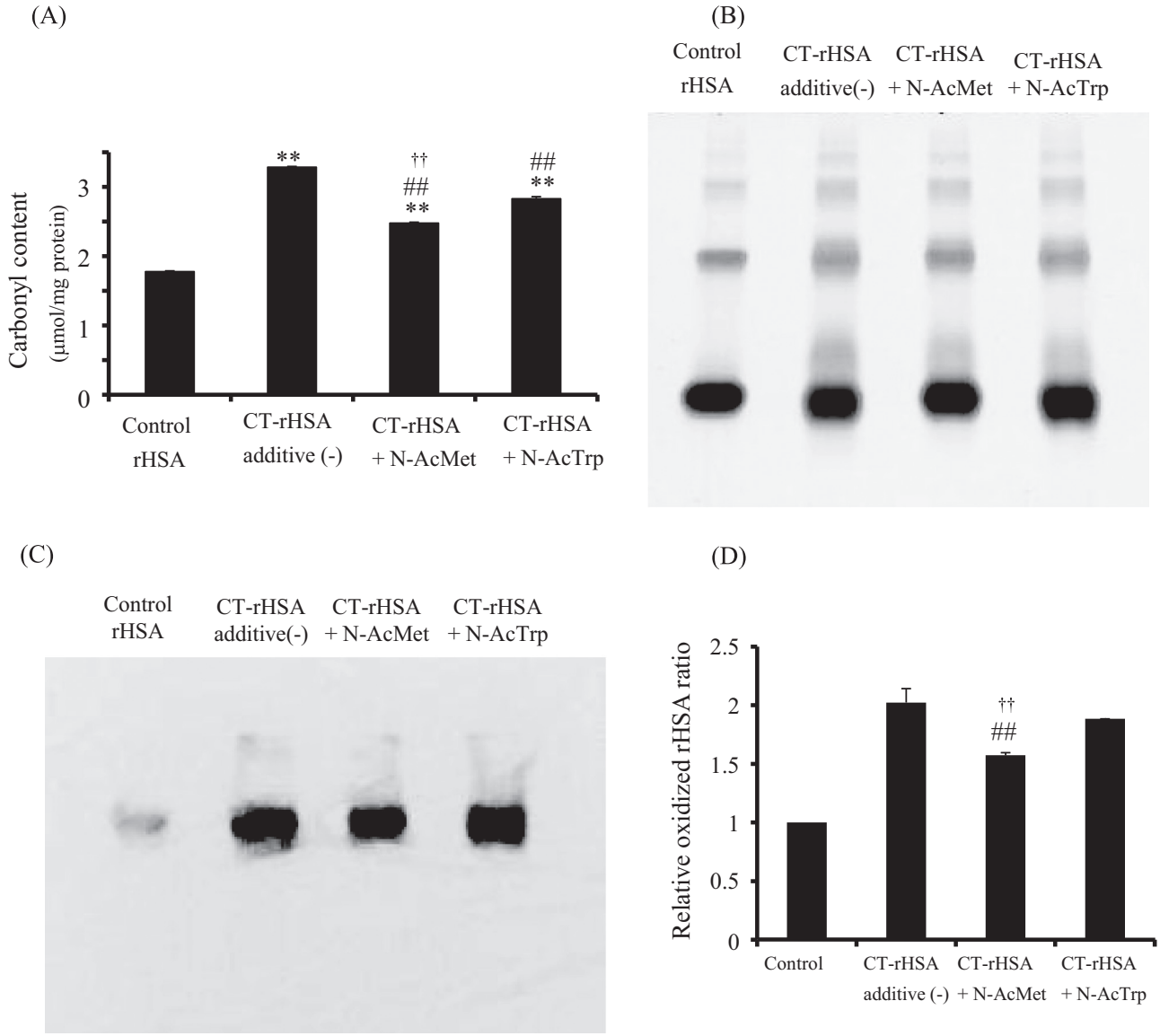

Fig. 2. (A) Carbonyl content of non-treated rHSA (control) and oxidized rHSAs. The concentration of rHSA was 0.2 mM and that of each additive was 1 mM. Each column represents the mean ± S.D. (n =3). $^{**}P < 0.01$, compared with control. $^{\#\#}P < 0.01$, compared with CT-rHSA additive (-). $^{\dagger\dagger}P < 0.01$, compared with CT-rHSA + N-AcTrp. (B) Native-PAGE electrophoresis. The concentration of rHSA was 0.1 mM and that of each additive was 0.5 mM. (C) Western blots and staining with Oxyblot Kit reagents. (D) Level of rHSA oxidation as determined by densitometry ratios between DNP area and corresponding protein area. Each column represents the mean ± S.D. (n=4). $^{\#\#}$ $P < 0.01$, compared with CT-rHSA additive (-). †† $P < 0.01$, compared with CT-rHSA + N-AcTrp.

3. Results

3.1. The level of rHSA oxidation without and with additives

3.1.1. AOPP contents

As seen in Fig. 1, treatment of rHSA with CT increases the AOPP content of the protein very much, i.e., from 5.3 ± 1.3 μM to 62.5 ± 2.3 μM ($p < 0.01$). The value obtained for the CT-treated albumin (non-additive CT-rHSA) corresponds to those of the proteins in uremic plasma [24]. This finding suggests that the state of non-additive CT-rHSA reflects the state of AOPP in uremia [25]. Oxidation in the presence of N-AcMet or N-AcTrp results in a decreased amount of AOPP. In particular, the protective effect of N-AcMet is pronounced, because the AOPP formed in its presence is diminished to 45% of that formed without additive ($p < 0.01$). The content is also significantly lower than that formed in the presence of N-AcTrp, which results in the formation of 57% of that formed without additive ($p < 0.01$).

3.1.2. Carbonyl contents

The extent of oxidation of rHSA after CT-induced oxidative stress was also evaluated by measuring the content of carbonyl groups by spectrophotometry after their derivatization with fluoresceinamine. As seen in Fig. 2A, N-AcTrp but especially N-AcMet has protective effects against oxidation, i.e., the carbonyl contents were diminished by 16% and 29%, respectively, as compared to the content measured without additive.

3.1.3. Native-PAGE

Fig. 2(B) shows the effect of oxidation on the migration and broadening of rHSA using native-PAGE. As seen, treatment of rHSA alone or in the presence of N-AcTrp effected the albumin migration and caused a clear broadening of the major protein bands. These effects were less pronounced, if the oxidation took place in the presence of N-AcMet.

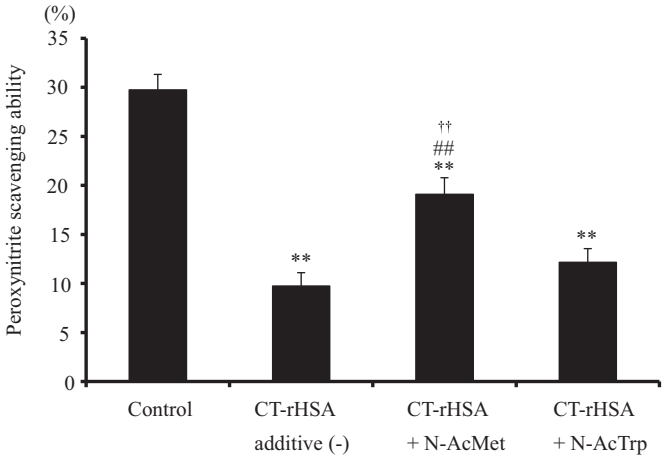

Fig. 3. Relative peroxynitrite scavenging activity of non-treated rHSA (control) and oxidized rHSAs. The concentration of rHSA was 25 μM. Each column represents the mean ± S. D. (n=3). ** $P<0.01$, compared with control. ## $P<0.01$, compared with CT-rHSA additive (-). †† $P<0.01$, compared with CT-rHSA + N-AcTrp.

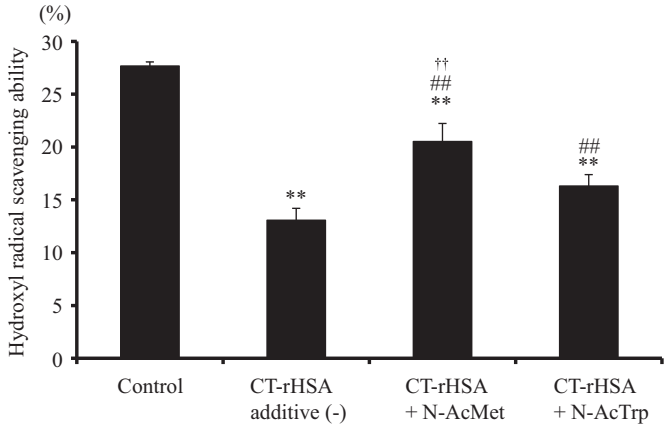

Fig. 4. Relative hydroxyl radical scavenging activity of non-treated rHSA (control) and oxidized rHSAs. The concentration of rHSA was 50 μM. Each column represents the mean ± S. D. (n=3). ** $P<0.01$, compared with control. ## $P<0.01$, compared with CT-rHSA additive (-). †† $P<0.01$, compared with CT-rHSA + N-AcTrp.

3.1.4. *Western blotting*

The carbonyl contents of oxidized rHSAs were also determined by Western blot analysis using an anti-DNP antibody (Fig. 2C). The content of carbonyl groups in untreated and in oxidized rHSA was calculated as the densitometry ratio between the DNP area and the corresponding protein area (see Section 2.3.1.). These carbonyl/protein ratios are regarded as relative oxidized rHSA ratios and are given in Fig. 2D. As seen, only CT-HSA with N-AcMet decreased slightly, but significantly, the carbonyl/protein ratio, whereas N-AcTrp had no significant effect on the ratio. Thus, taking all of the results in Fig. 2 into account, N-AcMet seems to be the most effective additive in protecting rHSA against oxidation.

3.1.5. *Peroxynitrite scavenging activity*

In this experimental set-up, peroxynitrite can have two effects. It can either oxidize albumin, or it can oxidize dihydrorhodamine 123 to rhodamine 123, the fluorescence of which is measured. Therefore, the better antioxidant, the higher peroxynitrite scavenging activity and the lower fluorescence. As seen in Fig. 3, the scavenging activity of CT-rHSA with N-AcMet is most pronounced of the three oxidized albumin preparations. This effect is observed, because the antioxidant properties of rHSA are best protected by N-AcMet.

3.1.6. *•OH scavenging activity*

ROS such as •OH lead to cell death and trigger various diseases related to oxidative stress. Therefore, the scavenging effect of the additives on •OH species was evaluated by means of an ESR spin-trapping method, namely by monitoring the intensity of ESR signals of DMPO-OH adducts produced by the reaction of DMPO with •OH. The ESR intensities of the DMPO-OH adduct were reduced by the addition of additives, indicating that •OH species were scavenged. The relative intensity of the DMPO-OH adducts compared to an external reference of Mn^{2+} was plotted to calculate the scavenging ability (%) of additives. As seen in Fig. 4, the •OH scavenging activity of CT-rHSA with N-AcMet was found to be superior to that of CT-rHSA with N-AcTrp and much higher than that observed for CT-rHSA without additive. As in the case of the peroxynitrite scavenging activity (see Section 3.1.5.) these findings are in accordance with the proposal that N-AcMet is a better protector against oxidation than N-AcTrp.

3.2. *Structural characteristics of rHSA oxidized without and with additives*

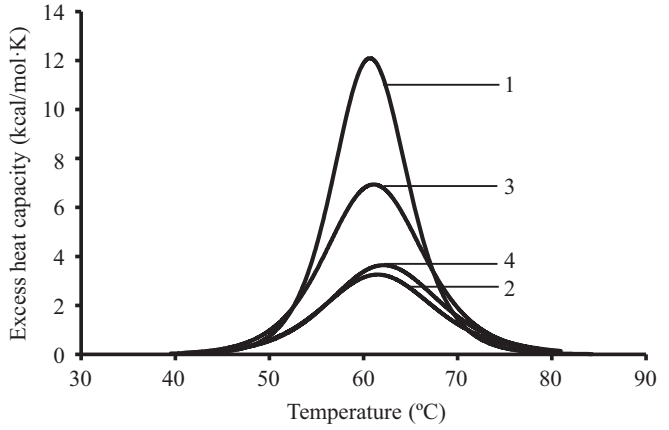

Fig. 5. Thermogram of non-treated rHSA (control) and oxidized rHSAs obtained by DSC. (1) control, (2) CT-rHSA additive (-), (3) CT-rHSA + N-AcMet, (4) CT-rHSA + N-AcTrp dissolved in sodium phosphate buffer, pH 7.4. The protein concentration was 20 μM.

Table 1
Thermodynamic data obtained from DSC of different rHSA samples after CT-induced oxidation.[a]

Protein samples	T_m (°C)	ΔH_{cal} (kcal/mol)	ΔH_v (kcal/mol)	$\Delta H_v/\Delta H_{cal}$
Control	60.8 ± 0.09	126 ± 3.0	83.8 ± 4.1	0.66 ± 0.20
CT-rHSA additives (-)	61.7 ± 0.07	48.2 ± 2.5**	56.9 ± 3.4**	1.18 ± 0.10
CT-rHSA + N-AcMet	61.2 ± 0.05	91.9 ± 4.9**	64.1 ± 2.9**	0.70 ± 0.18##, †
CT-rHSA + N-AcTrp	62.3 ± 0.10	54.3 ± 3.9**	57.4 ± 1.9**	1.06 ± 2.9

[a] The concentration of rHSA was 20 μM and that of each additive was 100 μM. The results are means ± S. D. (n=3).
** $P<0.01$, compared with control.
$P<0.01$, compared with CT-rHSA additive (-).
† $P<0.05$, compared with CT-rHSA + N-AcTrp.

3.2.1. *DSC-studies*

The thermal denaturation of rHSA oxidized in the absence and presence of an additive was examined by means of DSC measurements (Fig. 5). As quantitated in Table 1, the denaturation temperature (Tm) was not significantly affected by oxidation. The

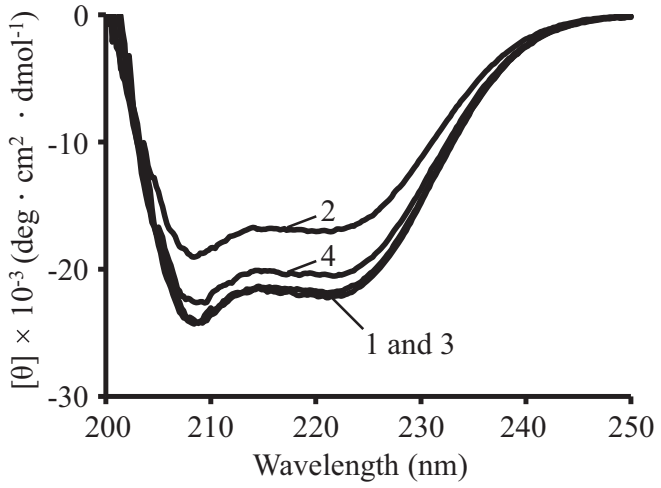

Fig. 6. Far-UV CD spectra of non-treated rHSA (control) and oxidized rHSAs. (1) control, (2) CT-rHSA additive (-), (3) CT-rHSA + N-AcMet, (4) CT-rHSA + N-AcTrp dissolved in sodium phosphate buffer, pH 7.4. The protein concentration was 10 μM.

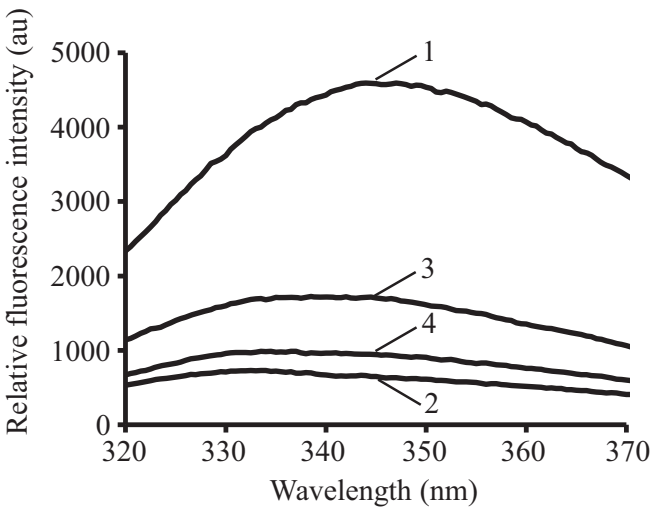

Fig. 7. Intrinsic fluorescence spectra of non-treated rHSA (control) and oxidized rHSAs. (1) control, (2) CT-rHSA additive (-), (3) CT-rHSA + N-AcMet, (4) CT-rHSA + N-AcTrp dissolved in sodium phosphate buffer, pH 7.4. The protein concentration was 2 μM. au, arbitrary units.

decreases in the ΔHcal-value suggest that the oxidized forms are more easily denatured, which again implies that the protein structures are more open (i. e. more hydrophobic regions are exposed to the solvent) at 25 °C. This suggestion was tested by using the fluorescence probe bis-ANS, which binds to hydrophobic areas. The results obtained with bis-ANS also strongly indicate the formation of additional accessible hydrophobic areas in rHSA oxidized in the presence of N-AcTrp. By contrast, only small changes take place in the case of rHSA oxidized in the presence of N-AcMet (data not shown). The denaturation enthalpies (ΔHv) were affected as follows: Control-rHSA > non-additive CT-rHSA ~ CT-rHSA with N-AcMet ~ CT-rHSA with N-AcTrp. The ΔHv/ΔHcal-values, and the corresponding broadening of the peaks in Fig. 5, indicate that, although the thermal denaturation of the protein forms takes place at the same temperature, the denaturation of rHSA + N-AcMet probably involves intermediary steps, because the ΔHv/ΔHcal-value is below 1. By contrast, denaturation of rHSA + N-AcTrp could involve some multimerisation of the protein, because the ΔHv/ΔHcal-value is slightly above 1. Thus, the DSC-studies suggest that the structural changes of rHSA are significantly smaller in the presence of N-AcMet than in the presence of N-AcTrp.

3.2.2. CD spectra

As can be seen in Fig. 6, CT-induced oxidation decreased in all examples the α-helical content of rHSA. The decrease was smallest for CT-rHSA with N-AcMet, namely 6.8%. In the other cases, the decreases for CT-rHSA with N-AcTrp and non-additive CT-rHSA, were 15.5% and 16.2%, respectively. These findings also suggest a superior role for N-AcMet in protecting rHSA during CT induced oxidation.

3.2.3. Effect on aromatic amino acid residues

The effect of oxidation on the single tryptophan residue in position 214 in albumin was monitored by fluorescence measurements (Fig. 7). As seen, CT-rHSA with N-AcMet resulted in the smallest decrement in the fluorescence, i.e., 48.2% at λ_{max}. In contrast, CT-rHSA with N-AcTrp and, especially, non-additive CT-rHSA had much lower fluorescence intensities, i.e., the decreases were 80.2% and 83.8%, respectively. Likewise, the shift of λ_{max} towards shorter wavelengths seems to be most pronounced in the latter two cases. The light absorbance spectra from 200 nm to 400 nm were affected in principally the same manner as found for the fluorescence spectra (not shown). The changes in the two types of spectra suggest that minor conformational changes have taken place in the vicinity of the tryptophan residue, and that the changes are most pronounced for CT-rHSA oxidized without or with N-AcTrp. In this connection it is of interest to note that native-PAGE of rHSA + N-AcTrp and of rHSA alone showed a more evident effect on albumin migration and on the width of the major protein bands (Fig. 2(B)). These indications of aggregate formation are in accordance with transfer of Trp214 to a more hydrophobic environment (Fig. 7).

3.3. Plasma half-lives of rHSA oxidized without and with additives

In an attempt to evaluate whether CT-induced oxidations without and with additives had any effect on the biological fate of rHSA, we determined plasma half-lives of the rHSAs in mice (Fig. 8). CT-rHSA with N-AcTrp ($t_{1/2}$=4.98 h) and non-additive CT-

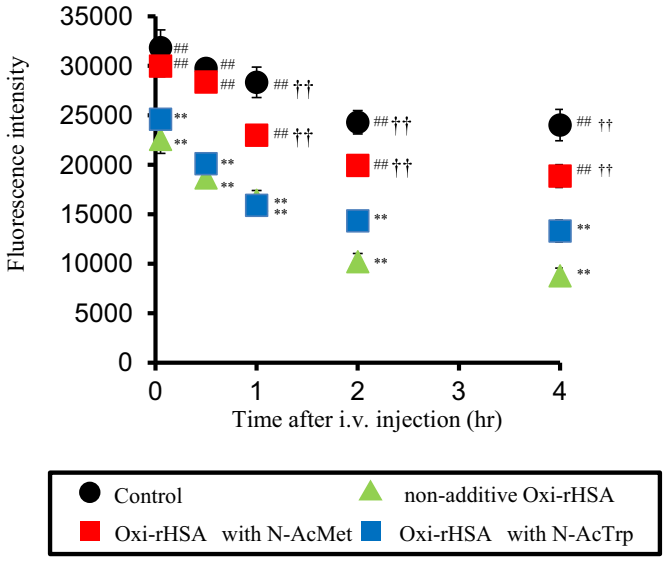

Fig. 8. In vivo pharmacokinetics of ICG-labeled, non-treated rHSA (control) and oxidized rHSAs. Each data point represents the mean ± S.D. (n=6). ** $P<0.01$, compared with control. ## $P<0.01$, compared with CT-rHSA additive (-). †† $P<0.01$, compared with CT-rHSA + N-AcTrp.

rHSA ($t_{1/2}$=2.86 h) showed shorter half-lives as compared with that of CT-rHSA with N-AcMet ($t_{1/2}$=5.87 h) and control-rHSA ($t_{1/2}$=9.65 h). Fluorescence measurements carried out with isolated organs showed that liver uptakes of CT-rHSA with N-AcTrp and non-additive CT-rHSA were much more pronounced than that of control rHSA and CT-rHSA with N-AcMet (not shown). This finding most probably explains the shorter half-lives of the two former preparations.

4. Discussion

The annual production of purified HSA for clinical purposes now exceeds 300 t [1]. In addition, rHSA with bound therapeutics are already used in the clinic, and rHSA, without or with other therapeutics, and albumin fusion proteins are soon available for clinical use. Therefore, it is surprising that only sparse information can be found about the mechanism of protection of the standard additives Oct and N-AcTrp during any pasteurization and during storage and about their potential adverse effects caused by their presence in the albumin products. Previously, we have studied the effects of these additives during pasteurization and found that Oct has a good stabilizing effect against heat, whereas the presence of N-AcTrp diminishes oxidation of HSA [10]. It is commonly accepted that albumin products with these additives can be stored at room temperature for as long as two years [1]. We have recently found that N-AcMet is a safe and risk-free stabilizer of albumin during photo-irradiation for long storage [12]. Actually, N-AcMet is superior to N-AcTrp in this respect, because exposure to light photo-degrades the latter to potentially toxic compounds which promotes photo-oxidative damage of albumin. However, it is not known whether oxidation can affect the quality of albumin products during long storage or upon administration, and how it is possible to diminish any such changes. In the present study we investigated the protective effects of N-AcMet, compared with those of N-AcTrp, and used CT as a model compound for mimicking oxidative stress.

Dependent on the molar ratio between CT and albumin, CT is able to oxidize with increasing efficiency Cys34, methionine residues, Trp214, arginine, lysine and proline residues in the protein [26–28]. In accordance with this, we found that CT-treatment of rHSA resulted in the formation of a pronounced content of AOPP (Fig. 1) and carbonyl groups (Fig. 2). The results also clearly showed that N-AcMet is a better protector against the CT-induced oxidation than N-AcTrp. Furthermore, the antioxidant properties of rHSA are best preserved, when the treatment with CT takes place in the presence of N-AcMet. Thus, this type of preparation is superior in scavenging peroxynitrite (Fig. 3) and the hydroxyl radical ($^{\bullet}$OH) (Fig. 4). Taking all of these results into consideration, N-AcMet is more efficient than N-AcTrp in protecting rHSA against oxidation. In this respect it is of interest to note that we have previously found that N-acetyl-cysteinate has no protective effect against oxidation, because N-acetyl-cysteinate interacts covalently with the thiol group of Cys34 [10].

What residues in rHSA are mostly affected by the CT-induced oxidation? Bourdon et al. [27] have reported that among the 585 amino acid residues in HSA, Cys34 and the methionine residues account for 40–80% of the total antioxidant activity of the protein. We have previously quantitatively evaluated the role of these residues for the antioxidative activity of rHSA using recombinant mutants, in which Cys34 and/or the six methionine residues had been mutated to alanine using site-directed mutagenesis [21,28]. The results showed that the level of contribution of Cys34 and the methionine residues to the antioxidative activity of rHSA was 61% and 29% against $O_2^{\bullet -}$, 68% and 61% against H_2O_2, 38% and 6% against $^{\bullet}$OH, 36% and 13% against HOCl and 51% and 1% against $^{\bullet}$NO, respectively. This information suggests that especially Cys34 but also the methionine residues are mostly involved in the present CT-induced oxidation, because CT oxidizes via the formation of $^{\bullet}$OH and chloro radicals [28].

Differential scanning calorimetry is a powerful method that provides a complete thermodynamic characterization of the stability of a protein as a function of temperature [29,30]. Therefore, to validate the benefit of N-AcMet as a structural stabilizer, we also studied the stabilizing effect of N-AcMet against CT-induced oxidation by DSC. We observed that the endotherms were single peaks, indicating that thermal denaturation can be explained by a single component model (Fig. 5) [22, 29–31]. Therefore, single values for Tm, ΔHcal and ΔHv could be calculated (Table 1). ΔHcal, which is generally thought to reflect the hydration of hydrophobic regions buried in the native protein structure during the unfolding process, is higher for rHSA oxidized in the presence of N-AcMet than in the presence of N-AcTrp. The ratio of ΔHv/ΔHcal is an index of the transition process to the denaturation states of proteins during thermal denaturation [32]. The value of this ratio for control-HSA was almost identical to that for CT-HSA with N-AcMet, whereas those for CT-HSA with N-AcTrp and non-additive CT-HSA increased. Thus, N-AcMet gave a greater protection against oxidative stress than N-AcTrp.

In an attempt to differentiate the conformational changes of the three oxidized rHSA preparations detected by DSC, we studied their CD, fluorescence and light absorbance spectra. The CD spectra revealed only a minor decrease in the α-helical content of rHSA, when oxidation took place in the presence of N-AcMet. By contrast, almost no protective effect was observed by N-AcTrp (Fig. 6). The fluorescence and light absorbance measurements also showed conformational changes in all three cases, but they were less pronounced if the CT-treatment took place in the presence of N-AcMet (Fig. 7 and not shown). The modified spectra reflect conformational changes involving Trp214, caused by oxidation of the residue itself or indirectly by oxidation of Cys34 and/or one or more methionine residues. In conclusion, N-AcMet is more useful than N-AcTrp as a structural stabilizer.

N-AcMet binds to one site on native rHSA with a low affinity, i.e., $K_{ass}=5.5\times10^4\ M^{-1}$ [12]. Thus, the protective effect of N-AcMet is not due to a strong binding, why it should also be useful as a stabilizer for other solubilized proteins.

The clearance of albumin depends on various factors including its degradation by a variety of organs, transcapillary escape, filtration and reabsorption, lymphatic drainage just to name a few. These pathways in turn are also influenced by the physicochemical state of the protein such as its charge and conformation. In skin, the clearances for the most neutral modified albumins and cationic albumins were found to be 20% and 80% greater than that for native albumin, respectively. In skeletal muscle, the clearances for the most neutral modified albumins and cationic albumins were found to be 50% and 1.5 times greater than that for native albumin, respectively. This clearly shows that charge affects the transvascular transport of albumin [33,34]. In addition, environmental factors such as the presence of nitric oxide have been reported to influence the extravasation of albumin [35]. We found that the rate of protein clearance from the blood also depends on the degree of oxidation (Fig. 8). One reason for the shorter half-life and increased liver uptake of oxidized HSA could be the induced conformational changes. In this connection, it is noteworthy that Iwao et al. [36] reported a correlation between α-helix contents and the in vivo degradation rates of modified albumins. In the present work, the CT-induced oxidation also results in a slight decrease in α-helix content of the protein (CT-rHSA additive (-) ($52.2\pm2.9\%$) < CT-rHSA with N-AcTrp ($52.8\pm2.35\%$) < CT-rHSA with N-AcMet ($61.5\pm3.21\%$) < control rHSA ($68.3\pm1.18\%$)). Furthermore, we also found a good linear relationship between half-

life and α-helix content of the protein ($r = 0.935$, $P < 0.01$) (data not shown). In other studies with genetic variants of HSA, Iwao et al. [37,38] found that the diminished half-lives of their HSA preparations were accompanied by increased liver uptake clearances. Principally the same observation was made in the case of the present preparations of oxidized rHSA (not shown).

Uptake of albumin by the liver is mainly due to the presence of cell membrane receptors which recognize the protein and then internalize it by endocytosis. Several possibilities seem to exist. Among the receptors is one which is engaged in the rapid uptake of oxidized albumin. Other possibilities are scavenger receptors such as gp18 and gp30, but it is at present only speculative whether or not the present albumin forms can interact with them. Finally, liver uptake by adsorptive endocytosis could be influenced by the amino acid changes, because this type of uptake is dependent on the net charge of the protein, which most probably is modified in the present albumin forms. However, the detailed mechanism for the liver uptake of oxidized albumin remains to be clarified.

In recent years, technology for the mass production of recombinant rHSA has been established [39]. Therefore, in the near future, highly stable and inexpensive rHSA stabilized by N-AcMet could be available for clinical and other applications.

5. Conclusion

Apparently, the roles of N-AcMet and N-AcTrp for the post-translational oxidation 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 has a good stabilizing effect on protein structure and on its anti-oxidant properties. Finally, the reduced oxidized rHSA burden helps maintain normal pharmacokinetics. Thus, N-AcMet should be useful as a new stabilizer and anti-oxidant for preparations of albumin and other solubilized proteins. We expect N-AcMet will be approved as a new stabilizer in the near future.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.04.011.

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