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Calorimetric and spectroscopic properties of small globular proteins (bovine serum albumin, hemoglobin) after free radical generation

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Abstract

Mild oxidation of –SH-containing proteins (serum albumin, hemoglobin) by Ce(IV)-ions in the presence of the spin trap phenyl-*tert*-butylnitrone (PBN) resulted in the appearance of strongly immobilized nitroxide free radicals which evidences the formation of thiyl radicals on the thiol site of the proteins. In hydroxyl free radical generating system a fraction of strongly immobilized nitroxide radicals was also detected in these proteins, which implies that the oxidation of a fraction of the thiol groups was also involved in the free radical reaction. According to the differential scanning calorimetry (DSC) experiments the melting processes of the proteins were calorimetrically irreversible, therefore the two-state kinetic model was used to evaluate the experiments. The results support the view that site-specific interaction of SH-containing proteins with hydroxyl and thiyl free radicals is able to modify the internal dynamics of proteins and affect the conformation of large molecules.
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Keywords: Bovine serum albumin; Hemoglobin; Differential scanning calorimetry; Spin trapping; Thiyl free radicals

1. Introduction

The oxidative damage of biomolecules has recently received a great attention because of its importance in understanding a variety of biological phenomena (aging process, cancer study, different diseases, endogenous toxins). These processes produce various types of free radicals which lead to different mechanisms [1–4,4a]. The generated free radicals can react rapidly with a range of targets including the side chains of amino acids [5,5a,6]. Due to the varied nature of the amino acid side chains different free radicals are formed depending on the environment of the side chains and on the different factors as ionic strength, pH and temperature. However, site-specific oxidation of the proteins allows to study the consequences of radical reaction on the protein conformation and internal dynamics.

A number of protein reactive compounds, including the thiol reagents, are able to modify the structure and dynamic of the proteins. Small –SH-containing compounds might play protective function, whereas membrane proteins and enzymes usually involved in metabolic and biosynthetic processes. The roles of sulfhydryl groups in biological systems are complex, but the blocking of the –SH groups of proteins can result in destabilization of the proteins and affect the biochemical reactions. It is reported by Hill and McAuley

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[7] and later Gilbert et al. [8] that Ce(IV) oxidises thiols and disulphides and during the reaction thiyl free radicals are formed. This reaction was successfully extended and applied to proteins by Graceffa [9]. It was also shown that cysteine reduces Cr(VI) to Cr(V) intermediate [10,11]. The latter intermediate can react with H_2O_2 and thereby hydroxyl free radicals are generated via a Fenton-like reaction. In addition, the reaction of Cr(VI) with glutathione generates Cr(V)–GSH complexes and $GS^{\bullet}$ radical, which can be identified by EPR and spin trapping methods. The two proteins involved in our experiments have a single sulfhydryl group (albumin at Cys-34, hemoglobin at Cys-93), which are located in a readily accessible small crevice. Their structure and dynamics are well known and therefore they have been used extensively as model proteins [12–14].

In this work we studied the effects of oxygen free radicals on specific sites of bovine serum albumin and hemoglobin in solution and in supramolecular complexes using spin trapping EPR technique. Serum albumin and hemoglobin are small globular proteins, which are essential components of the blood and were the targets of several experiments, because both of them have only one cysteine residue. Our results with spin trapping technique support the view that thiyl radicals can be generated in sulfhydryl-containing protein system by mild treatment. differential scanning calorimetry (DSC) measurements were performed to see whether the formation of free radicals on specific sites of the proteins could affect the global conformation. The kinetic theory developed recently seemed to be suitable to interpret the DSC measurements [20–24].

2. Materials and methods

2.1. Materials

Potassium chloride (KCl), magnesium chloride ($MgCl_2$), ethylenediamine-*N*,*N*′-tetraacetic acid (EDTA), *N*-ethyl maleimide (NEM), nitrilotriacetic acid (NTA), *tert*-butyl hydroperoxide (*t*-BuOOH), phenyl-*tert*-butylnitrone (PBN) and 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (MSL) were obtained from Sigma (Germany). Ammonium cerium(IV) nitrate was obtained from Merck (Germany). Albumin (BSA) and hemoglobin were purchased by Sigma (Germany). They were not further purified.

2.2. Preparation of proteins

2.2.1. Bovine serum albumin

A stock solution of 10–20 mg/ml (150–300 μM) BSA was prepared in 5 mM phosphate buffer pH 7.4 and exhaustively dialyzed overnight against the same buffer at 4 °C. In some cases 1 mM glutathione was added to the buffer solution to reduce the oxidized –SH groups. The samples were centrifuged at 200 1/s for 5 min before measurements in order to remove the insoluble particles.

2.2.2. Hemoglobin

Blood was obtained from healthy voluntary donors according to the requirement of University Ethic Committee. The blood samples were collected in EDTA-containing tubes and centrifuged for 20 min at 4000 × *g*. The plasma was removed and used immediately for EPR measurements. The red blood cells (RBCs) were resuspended in isotonic buffer solution containing 5 mM phosphate, pH 7.4. The RBCs were washed repeatedly in isotonic buffer solution before further treatments. The cell lysis was accomplished in hypotonic buffer solution (0.01 mM phosphate, pH 7.4). The samples were centrifuged at 30 000 × *g* at 4 °C and the supernatant was collected. The experiments were performed on freshly prepared hemoglobin. Before use the hemoglobin solution was centrifuged again to remove the rest of aggregated particles. The commercial hemoglobin was used to calculate the rotational correlation time of the attached spin adduct molecules in the proteins, which were used in the experiments.

The DSC measurements were done on hemoglobin samples prepared from lyophilized hemoglobin to ensure the required higher protein concentration.

2.3. Preparation of samples for EPR measurements

2.3.1. Generation of hydroxyl free radicals

OH radicals were generated by the metal ion-dependent breakdown of *t*-BuOOH. The composition of the reaction mixture was:

170 μl buffer solution or protein solution,
2 μl $FeSO_4$ (100 mM stock solution),

2 μl ATP (100 mM stock solution),
10 μl PBN (1 M stock solution),
1–3 μl *t*-BuOOH (77.6 mM stock solution).

The components of the reaction mixture were subsequently added to the buffer solution, the last component was *t*-BuOOH and then spectra were taken within 3–4 min. The preparation of samples was essentially the same as described earlier [15–17]. In some cases hydrogen peroxide was used instead of *t*-BuOOH.

2.3.2. Generation of thiyl free radicals

The Ce(IV)–nitrilotriacetic acid solution was prepared by adding one volume of 0.5 M $(NH_4)_2Ce(NO_3)_6$ solution to nine volume of 100 mM nitrilotriacetic acid, 0.5 M Tris–HCl, pH 8.25, following the method of Graceffa [9]. The concentration of the stock solution of PBN was 1 M. The samples for EPR measurement contained 2.5 mM Ce(IV)–NTA and 50 mM PBN, the protein concentration varied between 20 and 40 μM. Samples were prepared fresh, since, on standing, the radical concentration rapidly decreased with a half-time of $T_{1/2} = 30$ min. In samples of hemoglobin the final concentration of Ce(IV)–NTA was 3 mM.

2.4. DSC measurement

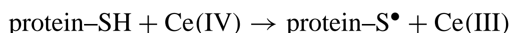
Thermal unfolding of albumin (10–20 mg/ml) and hemoglobin (10 mg/ml) was monitored by a SETARAM Micro DSC-II (SETARAM, Lyon, France) calorimeter. All experiments were done between 5 and 80 °C. The scan rate was 0.3 K/min. Conventional Hastelloy batch vessels were used during the denaturation experiments with 850 μl sample volume in average. Phosphate buffer was used as reference sample. The sample and reference vessels were equilibrated with a precision of ±0.1 mg. There was no need to do any correction from the point of view of heat capacity between sample and reference vessels. The samples were irreversible denatured during each cycle. The repeated scan of denatured sample was used as baseline reference, which was subtracted from the original DSC curve. Calorimetric enthalpy was calculated from the area under the heat absorption curve using two-point setting, SETARAM peak integration. The temperature and enthalpy calibration was done according to the SETARAM protocol.

2.5. EPR measurements

Conventional EPR spectra were taken with an ESP 300E (Bruker Biospin, Rheinstetten, Germany) X-band spectrometer. First harmonic, in-phase, absorption spectra were obtained using 20 mW microwave power and 100 kHz field modulation with amplitude of 0.1 or 0.2 mT at ambient temperature. For all spectra, sweep width was 10 mT, sweep time 81 s, time constant 20.4 ms, conversion time 41 ms and 10 scans were accumulated. The samples were placed in a flat quartz cell (Scanlon Co., USA, capacity about 80 μl), and spectra were recorded immediately after mixing the components. In some cases, due to the lower concentration of immobilized nitroxide free radicals, a larger flat cell with a capacity of about 700 μl was used. The instrument was calibrated with potassium peroxylamine disulphonate which has an $a_{iso} = 1.302$ mT in saturated bicarbonate solution. The spectra were normalized to the same number of unpaired electrons calculating the double integrals of the derived spectra, and the manipulations were performed on normalized EPR spectra. The concentration of free radicals in different samples was obtained by comparing the double integrals with that of a MSL solution of 10 μM concentration using the same sample holder and spectral parameters.

3. Results and discussion

Reaction of –SH-containing compounds and proteins with Ce(IV)-ions in the presence of spin trap PBN results in the appearance of nitroxide free radicals. Ce(IV) complexed to NTA oxidizes sulfhydryl compounds via thiyl free radicals, which can be trapped by PBN [9]:



$$\text{protein–SH} + \text{Ce(IV)} \rightarrow \text{protein–S}^\bullet + \text{Ce(III)}$$

$$\text{protein–S}^\bullet + \text{PBN} \rightarrow \text{protein–S–PBN}^\bullet$$

Similar experiments were done on small thiol-containing compounds using Cr(VI)-ions. The reaction with reduced glutathion generated Cr(V)–glutathion complex and PBN–GS$^\bullet$ spin adduct [18]. The singlet line detected at $g \cong 1.985$ corresponds to the Cr(V)–glutathion complex.

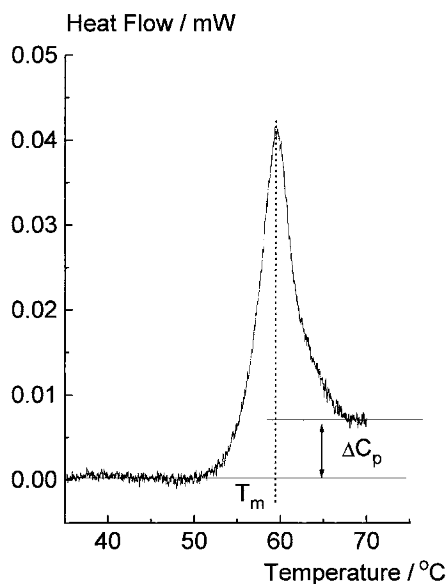


Fig. 1. DSC patterns of 300 μM BSA solution in 5 mM phosphate buffer at pH 7.4. The scan rate was 0.3 K/min.

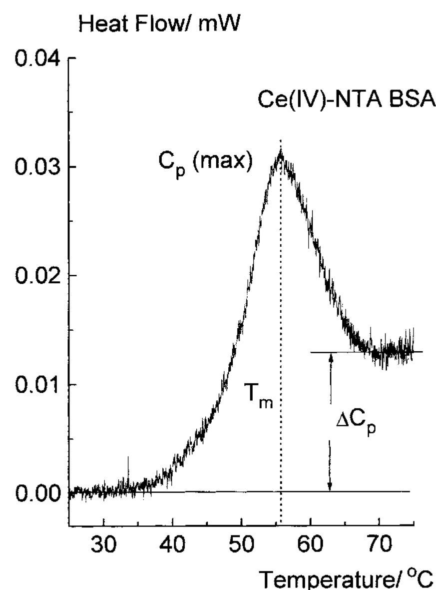
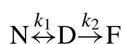


Fig. 2. DSC transition curve of 300 μM BSA solution after Ce(IV)–NTA treatment in 5 mM phosphate buffer at pH 7.4. The free radical damage on protein resulted in broadening of the DSC pattern. The scan rate was 0.3 K/min.

3.1. DSC measurements

Figs. 1 and 2 show the differential scanning calorimetry traces for the thermal denaturation of natural and Ce(IV)–NTA treated BSA solution. The large difference in the slopes of the pre- and post-transition base lines (see ΔC_p values in Figs. 1 and 2) might cause error in the determination of the thermodynamic parameters. The narrow peak ($\Delta T_m = 4.1\,°C$, the width of the melting curve at half-height) detected at $57.0 \pm 1.0\,°C$ ($n = 5$) in the absence of Ce(IV)–NTA complex showed after Ce(IV)–NTA treatment a large broadening ($\Delta T_m = 14.6\,°C$). There was only a small increase in the single transition temperature ($T_m = 57.6 \pm 1.0\,°C$, $n = 4$); the difference between the transition temperatures was not statistically significant.

We have found that, under certain conditions (pH, ionic strength) the DSC transitions were calorimetrically irreversible. The reversibility of the denaturation was checked by comparing the first scan of the sample with the second one after cooling the sample to room temperature. The simplest model, which can be applied, was first employed by Lumry and Eyring [19]. This theory assumes a reversible unfolding of the protein that is followed by a rate-limiting irreversible step:



$$\mathrm{N} \overset{k_1}{\leftrightarrow} \mathrm{D} \overset{k_2}{\rightarrow} \mathrm{F}$$

where F means the final state of the irreversible denatured protein, and the extent of the irreversibility is determined by the rate k_2 of the D → F step. The reaction rate depends on time and temperature. It was shown by Sanchez-Ruiz and co-workers that the two-state irreversible model describes fairly well the denaturation of thermolysin, carboxipepdidase and yeast phosphoglicerate kinase [20–22]. However, in some cases the thermodynamic parameters can be deduced from the standard treatment of the heat capacity curves [23,24].

As is evident in Fig. 3 that the transition temperature depends on the scan rate, therefore it can be assumed that the melting of the albumin is determined by kinetic processes, and can be described by the two-state kinetic model. From the assumption that the first-order rate constant follows the Arrhenius equation, the k_{app} values can be calculated from the relationship:

$$k_{app} = \frac{s_r C_p}{\Delta H_t - \Delta H}$$

where s_r is the scan rate, C_p means the apparent heat capacity, ΔH the excess enthalpy change and ΔH_t

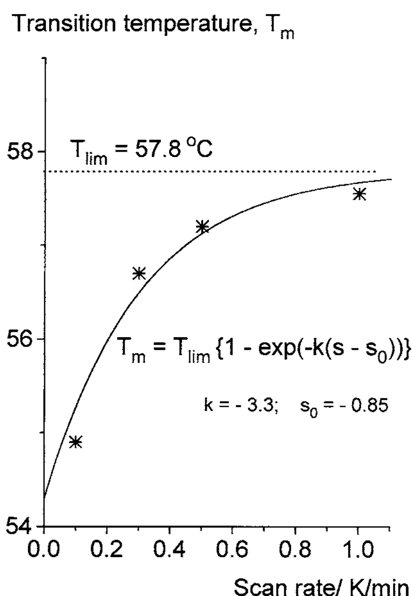


Fig. 3. Scanning rate effect of the DSC transitions for the melting process of native BSA solution. The concentration of the BSA was 300 μM. T_{lim} is the limiting value of the transition temperature, whereas k and s_0 are the parameters of the equation to obtain optimal fitting.

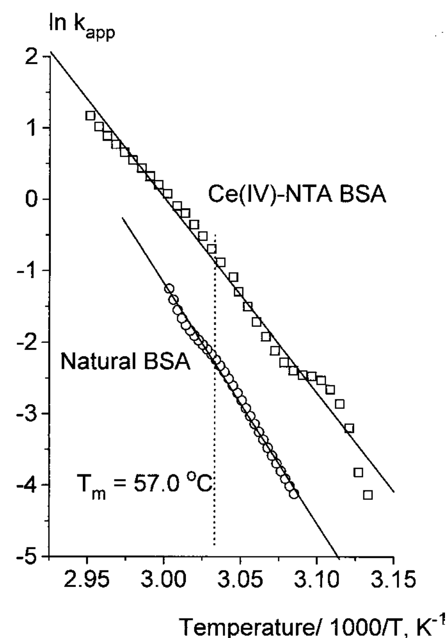


Fig. 4. Arrhenius plots of the first-order rate constants are plotted vs. $1/T$ for native and Ce(IV)–NTA treated BSA. The concentration of the BSA was 300 μM in both samples and the scan rate was 0.3 K/min. The activation energies were 273 and 230 kJ/mol, respectively.

is the total enthalpy change of the melting process [21]. Fig. 4 shows the Arrhenius plots of the native and the Ce(IV)–NTA treated BSA samples. In both cases, the plot of $\ln k_{app}$ versus $1/T$ could be approximated by a straight line, which implies that the kinetic model can be used to describe both melting processes. The slope of the regression line E_a/R gives the activation energy E_a (R is the gas constant), we calculated $E_a = 280 \pm 4$ kJ/mol (native BSA) and 230 ± 6 kJ/mol (Ce(IV)–NTA treated BSA), respectively. The kinetic theory also suggests that $\ln\{\ln[\Delta H_t/(\Delta H_t - \Delta H)]\}$ as a function of the reciprocal absolute temperature gives also a straight line (Fig. 5). The average activation energy from the plot thus obtained was 266 ± 5 kJ/mol.

The large increase of the width of the melting curve supports the view that the secondary reactions of the thiyl radicals affected significantly the protein structure, which led to the decrease of the cooperativity between the subunits during the melting process. This change might be accompanied with an increase of the internal flexibility. Similar effect was observed on hemoglobin samples. An alternative explanation might be that the excess heat capacity curve is the superposition of two melting curves, the first one represents an unaffected population, whereas the second one is probably due to the effect of modification of the protein structure produced by the thiyl free radicals and secondary reactions.

3.2. EPR experiments on BSA

The measurements on BSA and blood serum in thiyl free radical generating system showed the appearance of an EPR signal from strongly immobilized PBN spin adduct (Figs. 6 and 7B). The hyperfine splitting constant was $2A'_{zz} = 6.354 \pm 0.133$ mT (S.D., $n = 5$). It can be argued that as a consequence of the reactions in protein systems thiyl free radicals can be formed on the sulfhydryl groups, which were trapped by PBN [9,25]. Non-protein sulfhydryls cannot give EPR signals arising from strongly immobilized adduct molecules. Using NEM–BSA, the EPR signal intensity was markedly reduced. In our experiments BSA (147 μM) was pre-blocked with 20-fold

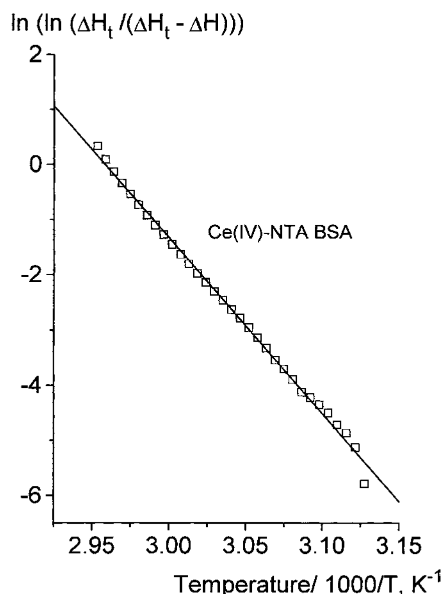


Fig. 5. Values of $\ln\{\ln[\Delta H_t/(\Delta H_t - \Delta H)]\}$ plotted vs. $1/T$ for Ce(IV)–NTA treated BSA. The concentration of the BSA was 300 μM, the scan rate was 0.3 min/K. The calculated activation energy was 263 kJ/mol.

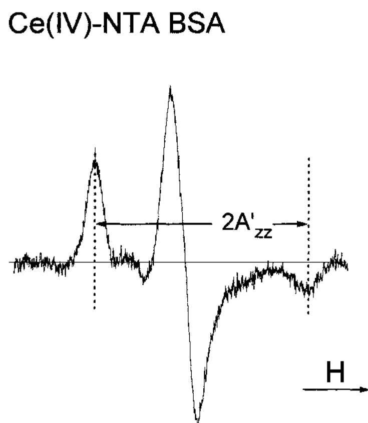


Fig. 6. EPR spectrum of BSA (147 μM) solution as a function of the static magnetic field (H) following treatment with Ce(IV)–NTA in the presence of PBN. Spectra were taken immediately after thiyl free radical generation. The field scan was 10 mT. The hyperfine splitting constant $2A'_{zz}$ is a characteristic spectral parameter, its value is related to the rotational mobility of the attached spin adduct.

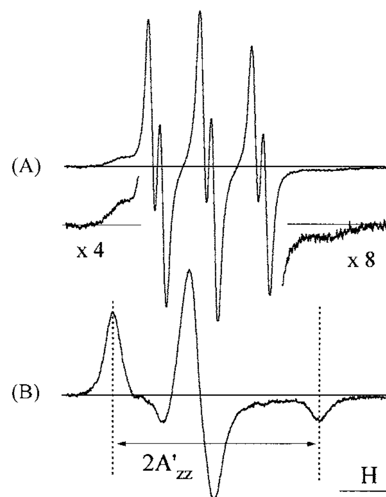


Fig. 7. EPR spectrum of blood serum in hydroxyl free radical generating system in the presence of PBN. The appearance of strongly immobilized nitroxide free radicals shows the formation of thiyl radicals on albumin after secondary reactions, which were trapped by PBN (A). After treatment with Ce(IV)–NTA only the strongly immobilized spectrum component was observed (B). The field scan was 10 mT.

molar excess of NEM for 24 h at 0 °C. Following this pretreatment, the protein sample was dialyzed overnight against phosphate buffer at 4 °C. The double integral of the sample significantly lowered a value of 44% of the untreated sample. This result evidences that the single cysteine (Cys-34) is involved in the immobilized adduct formation. MSL attached to the sulfhydryl group of bovine serum albumin resulted in a value of 6.501 ± 0.035 mT ($n = 3$). The difference between the obtained hyperfine splitting constants is probably due to the different attaching linkage of MSL and PBN.

The measurements on BSA and blood plasma in hydroxyl free radical generating system also showed the appearance of an EPR signal from a fraction of strongly immobilized PBN spin adduct [26]. The hyperfine splitting constant was the same value as in Ce(IV)–NTA system in the limits of the experimental error, indicating the presence of trapped PBN spin adducts. The fraction of strongly immobilized paramagnetic species appearing in the EPR spectra of proteins in hydroxyl free radical generating systems might have different origins. The blocking of thiol groups resulted in reduced concentration of strongly immobilized PBN spin adduct molecules.

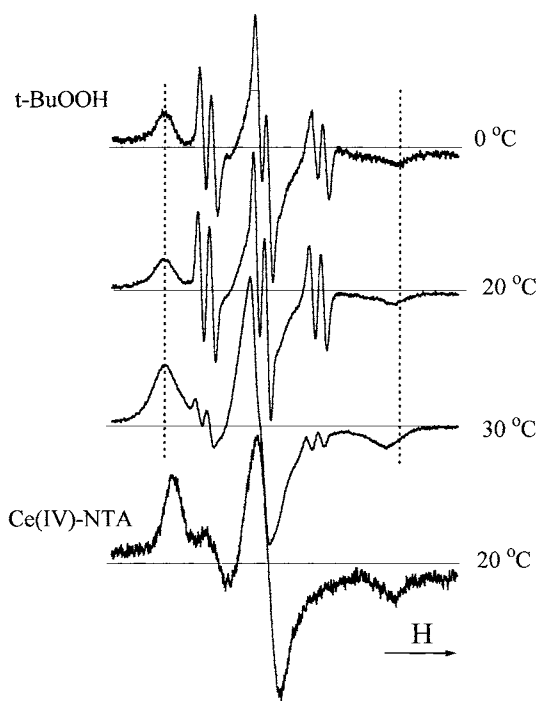


Fig. 8. EPR spectra of hemoglobin in hydroxyl and thiyl free radical generating systems. The formation of strongly immobilized spin traps depends strongly on temperature. In Ce(IV)–NTA system only strongly immobilized PBN molecules were detected.

This suggests a remarkable contribution from the thiol sites of the protein. The amount of strongly immobilized paramagnetic molecules can be overestimated by the non-specific binding properties of PBN spin adduct to the proteins. Albumin has many hydrophobic areas, and therefore it binds to small molecules as steroid hormones and fatty acids that are only slightly soluble in the blood serum [27].

3.3. Measurements on hemoglobin

The experiments were always performed on freshly prepared human hemoglobin, because, on standing, changes in the hyperfine splitting constant of the bound nitroxide free radicals and larger spread in the free radical concentrations were detected.

In thiyl free radical generating system, only the spectrum arising from strongly immobilized nitroxide free radicals was observed (Fig. 8, bottom spectrum). The concentration of the attached PBN spin adduct was $0.42 \pm 0.06\,\mathrm{M}$ ($n = 5$) of free radical/mol of hemoglobin. The estimation of the hyperfine splitting constant resulted in a value of $2A'_{zz} = 6.392 \pm 0.016\,\mathrm{mT}$ ($n = 6$), which was about the same as the value obtained on MSL–hemoglobin ($2A'_{zz} = 6.472\,\mathrm{mT}$). It suggests that the PBN spin adduct molecules located on the same site as the MSL molecules.

The spectra in hydroxyl free radical generating system exhibited the superposition of two spectral components. The ratio of fractions of the free radicals depended strongly on temperature. The total concentration of free radicals was $17.3 \pm 2.5\,\mu\mathrm{M}$ ($n = 5$) at room temperature. This value is larger than the concentration of spin adducts in thiyl free radical generating system, where $12.2 \pm 3.5\,\mu\mathrm{M}$ ($n = 4$) was obtained, using the same protein sample. The hyperfine splitting constant of the strongly immobilized PBN spin adducts was $2A_{zz} = 6.406 \pm 0.03\,\mathrm{mT}$ ($n = 4$) at room temperature, very similar to Ce(IV)–NTA hemoglobin.

4. Conclusions

Selective attack by mild oxidation at particular amino acid residues (Cys-34) on bovine serum albumin, blood serum and hemoglobin (Cys-93) provided evidence, that the free radical damage led to conformational changes, which affected the thermodynamic properties of the proteins. The most significant change was the broadening of the melting curve.

Random attack of hydroxyl free radicals on the proteins in solution and in blood serum modifies the internal structure of proteins, a fraction of the sulfhydryl groups is involved in the free radical reaction. The secondary reactions following the generation of free radicals might cause damage in the protein structure that might have consequences in the biochemical reactions.

Acknowledgements

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