Hydrogen Peroxide-induced Structural Alterations of RNase A*

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Proteins exposed to oxidative stress are degraded via proteolytic pathways. In the present study, we undertook a series of in vitro experiments to establish a correlation between the structural changes induced by mild oxidation of the model protein RNase A and the proteolytic rate found upon exposure of the modified protein toward the isolated 20 S proteasome. Fourier transform infrared spectroscopy was used as a structure-sensitive probe. We report here strong experimental evidence for oxidation-induced conformational rearrangements of the model protein RNase A and, at the same time, for covalent modifications of amino acid side chains. Oxidation-related conformational changes, induced by H₂O₂ exposure of the protein may be monitored in the amide I region, which is sensitive to changes in protein secondary structure. A comparison of the time- and H₂O₂ concentration-dependent changes in the amide I region demonstrates a high degree of similarity to spectral alterations typical for temperature-induced unfolding of RNase A. In addition, spectral parameters of amino acid side chain marker bands (Tyr, Asp) revealed evidence for covalent modifications. Proteasome digestion measurements on oxidized RNase A revealed a specific time and H₂O₂ concentration dependence; at low initial concentration of the oxidant, the RNase A turnover rate increases with incubation time and concentration. Based on these experimental findings, a correlation between structural alterations detected upon RNase A oxidation and proteolytic rates of RNase A is established, and possible mechanisms of the proteasome recognition process of oxidatively damaged proteins are discussed.

The close relationship between protein oxidation and susceptibility to proteolysis was documented in a series of publications from our laboratory and other groups (1–6). These studies were conducted with erythrocytes and reticulocytes of various species (7–9), with Escherichia coli (10), with primary and permanent cell culture systems (5, 6, 11–13), and with purified proteins and proteases (7, 8, 14, 15). A number of publications demonstrated the enhanced degradation of proteins oxidized by exposure to hydrogen peroxide, to the superoxide anion radical (O₂⁻), the hydroxyl radical (OH•), or to peroxynitrite (ONOO⁻) (1–6, 15). Most of these studies revealed that the degradation of mildly oxidized proteins seems to be a normal cellular function, whereas extensively oxidized proteins are poor substrates for proteases and may accumulate and therefore contribute to diseases or aging processes (16–18).

In studies using mammalian cells, cell lysates or extracts, strong evidence was presented for the key role of a single proteolytic complex, the 20 S proteasome, in the selective recognition and degradation of oxidatively damaged proteins (5, 6, 14, 19). The basis of this recognition still remains unknown. However, some experimental evidence suggests the selective recognition of hydrophobic moieties at the protein surface (14, 16, 20–22) or the importance of the methionine oxidation product, methionine sulfoxide (14, 21). The groups of Davies (16, 20) and Stadtman and Levine (14, 21) demonstrated several times the enhanced proteolytic susceptibility of oxidized proteins with increased surface hydrophobicity using various methods such as separation on hydrophobic interaction chromatography (16, 20) or the 8-anilino-1-naphthalenesulfonic acid-dependent fluorescence intensity (21). Levine et al. (14) demonstrated a correlation between methionine sulfoxide formation and proteolytic degradation. It was concluded that the oxidative modification of amino acid side chains disrupts, at least locally, the tertiary protein structure, which is, in turn, accompanied by exposure of hydrophobic moieties to the surface of the protein. This increase in surface hydrophobicity seems to be the recognition signal for the 20 S proteasome for binding and degradation of the substrate protein (14, 16, 18, 20–22). However, until now it could not be shown directly that protein oxidation is accompanied by the disruption of secondary and tertiary protein structure.

The present investigation addresses the question of whether protein oxidation is followed by amino acid side chain modifications or changes in secondary and tertiary protein structure and whether these effects correlate with increased proteolytic susceptibility. For our studies, we selected the small single domain cytoplasmic protein RNase A as a model, since this protein is exceptionally well characterized by a variety of structure-sensitive techniques such as x-ray crystallography (23), NMR spectroscopy (24), UV-visible and CD spectroscopy (25), differential scanning calorimetry (for a review, see Pace et al. (26)), and Fourier transform infrared (FT-IR) spectroscopy (27–30). In particular, FT-IR spectroscopy has proved to be a sensitive tool for following conformational changes in proteins (Ref. 31; for a review, see Jackson & Mantsch (32)). Peptide
backbone and side chain infrared “marker” bands can be employed as conformation-sensitive monitors to derive structural parameters during refolding of RNase A in solution (33, 34). Recently, time-resolved infrared spectroscopic techniques have been employed to follow refolding processes of RNase A in the time range of 50 ms to 15 minutes (29, 35, 36).

In the present paper, we report structural alterations, detected by FT-IR spectroscopy, of the model protein RNase A upon exposure toward hydrogen peroxide and upon thermal unfolding. The changes of the protein structure were related to the results obtained by degradation measurements of the oxidized RNase A samples by isolated 20 S proteasomes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Highly purified lyophilized RNase A (from bovine pancreas) was purchased from Sigma Chemie GmbH (Deisenhofen, Germany).

**Proteasome Preparation**—Proteasome was isolated from erythrocytes of outdated human blood conserves according to Hough et al. (37). Erythrocytes were lysed in 1 mM dithirotetrool. After the removal of membranes and nonlysed cells by centrifugation, the proteasome was isolated by DEAE chromatography, sucrose density gradient ultracentrifugation, and separation on a Mono Q column of a fast protein liquid chromatography system.

**RNase A Treatment with Hydrogen Peroxide**—All RNase A treatments were performed under standardized conditions to ensure reproducibility of the FT-IR spectroscopic and proteasome degradation experiments. Stock solutions containing RNase A were prepared in a 10 mM cadycyld/NaOH buffer at pH 7.1 to yield an enzyme concentration of 20 mg/ml. The protein solutions were heated for 15 min at 60 °C to achieve complete H/D exchange of the amide protons (28). The different H2O2 solutions (0, 10, 20, 40, and 80 mM) were prepared from a 22 mM H2O2 stock solution by dilution with the respective volume of a 10 mM cacodylat/D2O buffer, pH 7.1. The protein and H2O2 solution were subsequently mixed in a ratio of 1:1 (by volume) to achieve final protein concentration of 10 mg/ml in H2O2 solutions of 0, 20, 40, and 80 mM.) The latter value was estimated on the basis of the bond length and the atom radii of O2 and F2. We utilized an output file containing summed LeuCat surface maps of LeuCat-exchange of the amide protons was obtained atom radii of O2 and F2. We utilized an output file containing summed LeuCat surface maps of LeuCat-exchange of the amide protons was obtained after the first heating cycle. For data evaluation, only spectra of the second heating run (unfolding) were utilized. Infrared spectra were corrected for spectral contributions of buffer and water vapor as described previously (33). Difference spectra were calculated in the same way as outlined above.

**Calculation of the Accessible Surface Area**—The accessible surface areas (ASA) were calculated using the program Naccess version 2.1 (38), which is an implementation of the Lee and Richards method (39).

The program calculates the atomic accessible surface defined by rolling a probe of given size around a van der Waals surface. A slice thickness of 0.05 Å and a probe size of 1.4 Å for H2O and 2.1 Å for H2O2 was used. The latter value was estimated on the basis of the bond length and the atom radii of O2 and F2. We utilized an output file containing summed LeuCat-exchange of the amide protons after the first heating cycle. For data evaluation, only spectra of the second heating run (unfolding) were utilized. Infrared spectra were corrected for spectral contributions of buffer and water vapor as described previously (33).

**RESULTS**

To obtain new insights into the recognition process of oxidized proteins by the proteasome, we combined two different techniques: structural changes of the model protein RNase A upon oxidation by hydrogen peroxide were followed by FT-IR spectroscopy, while the changes of susceptibility to proteasome degradation were tested by biochemical methods. For this purpose, stock solutions of RNase A and H2O2 were prepared and used for both kinds of experiments. Although the use of D2O buffers or fully H/D-exchanged proteins is not essential for the proteasome degradation characterization, we tried to maintain the experimental protocols as much as possible. Therefore, the first steps of proteasome digestion were performed in D2O buffers after a complete H/D exchange of the protein.

**Proteolytic Susceptibility of RNase A**—The degradation of various proteins by the 20 S proteasome was already measured by several groups for a number of different proteins (5, 14, 15). Since most of these experiments were performed under standardized conditions, we used the same experimental protocol to study the proteolytic susceptibility of RNase A toward the 20 S proteasome. The results of the investigations are demonstrated in Fig. 1. When RNase A is treated with hydrogen peroxide concentrations up to 4 μmol/mg of protein (approximately 55 molecules of H2O2 per molecule of RNase A), an increase in proteolytic susceptibility of the resulting oxidized proteins is detected. At higher concentrations of hydrogen peroxide, a decrease of the proteolytic susceptibility was measured. As previously postulated and shown earlier (5, 13, 14, 41, 42), this decline is due to the irreversible formation of protein aggregates. These protein aggregates are poor substrates for the protease or may even be able to inhibit the 20 S proteasome (15, 18, 41, 42). One of the objectives of the present work was to investigate the structural rearrangements of the protein induced by oxidation, which causes the recognition and the deg-
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Fig. 1. Degradation of hydrogen peroxide-treated RNase A by the 20 S proteasome. RNase A (1 mg/ml) was treated with the indicated concentrations of hydrogen peroxide for 2 h and then extensively washed by ultrafiltration and incubated with the isolated 20 S proteasome. Proteolysis was measured by analyzing the acid-soluble supernatant for free amines. The data represent the mean ± S.D. of three independent experiments.

Fig. 2. FT-IR spectra of native RNase A at a temperature of 30 °C. The dotted curve displays an FT-IR spectrum of RNase A (buffer subtracted), while the solid curves were obtained applying "resolution enhancement techniques" to the original data (curve A, Fourier self-deconvolution; curve B, second derivative spectrum). The amide I region of RNase A spectra (1690–1620 cm⁻¹) is dominated by band components at 1631 and 1680 cm⁻¹, both assigned to antiparallel β-pleated sheets. Other amide I band components at 1665 cm⁻¹ (unordered structure) and 1651 cm⁻¹ (assigned to α-helical structures) are indicated. Absorptions of amino acid side chains at 1515 cm⁻¹ (tyrosine ring vibration) and near 1584 and 1566 cm⁻¹ (C=O vibrational modes of the aspartate and glutamate residues), although less intense than the amide I band, can be used to obtain additional structural information. AU, absorbance units.

The interpretation and quantification of structural changes of RNase A during oxidation by hydrogen peroxide was carried out, comparing these data with infrared spectra for thermally unfolded protein. It is known that RNase A can be reversibly unfolded thermally, giving rise to a characteristic spectral unfolding pattern, particularly in the amide I region (29, 33, 35, 43). In the present study, FT-IR spectra were collected between 20 and 80 °C, applying two consecutive heating and cooling cycles with a linear temperature gradient of 0.5 K/min. The FT-IR spectrum of native RNase A is shown in Fig. 2. This spectrum was recorded at a temperature of 30 °C after one heating and one cooling cycle (i.e., after complete thermal unfolding and refolding), which accelerates the exchange of the amide protons by deuterons (in the following, this procedure is called H/D exchange). The dotted line in Fig. 2A displays the absorbance spectrum corrected for the spectral contributions of the buffer, while the solid line represents the corresponding Fourier self-deconvolution spectrum (Fig. 2A) to demonstrate the fine structure of the amide I band. A second derivative spectrum (Fig. 2B) was also calculated from the absorbance spectrum (positive bands of an absorbance spectrum appear in second derivative spectra as negative bands). From the literature, it is known that the energy of the C=O oscillators of the protein backbone depends on the coupling to adjacent C=O oscillators and the strength of the hydrogen bonds. Furthermore, the strength of these bonds and the symmetry of hydrogen bond patterns is characteristic for distinct secondary structure elements of proteins. It is therefore possible to distinguish various secondary structures from the experimentally observed band components of the amide I band. For RNase A, the band assignment was carried out according to literature data (27, 29, 43, 44). In good agreement with these studies, IR marker bands for antiparallel β-pleated sheets were found at 1631 and 1680 cm⁻¹, for α-helix at 1651 cm⁻¹, and for unordered turn structures at 1665 cm⁻¹ (cf. Fig. 2). Also, infrared absorption bands of the amino acid side chains such as the "tyrosine band" (aromatic tyrosine ring vibration at 1515 cm⁻¹) or absorptions of aspartate and glutamate residues at 1584 and 1566 cm⁻¹, respectively, were observed in agreement with previous studies (45, 46).

Spectroscopic parameters of the amino acid side chain absorption bands can be utilized to derive structural information on the specific microenvironment of these functional groups. For example, the frequency of the tyrosine band at 1515 cm⁻¹ was used to monitor specifically the formation of tertiary contacts upon refolding (29). Fig. 3A displays a series of Fourier self-deconvolution infrared spectra (corrected for buffer) obtained by a linear temperature gradient measurement. As previously described for RNase A (29, 43), the appearance of a broad and featureless amide I band suggests the lack of stable secondary structure elements at temperatures above 70 °C. A frequently used approach to illustrate temperature-induced spectral changes is given in Fig. 3B, which shows a series of FT-IR difference spectra. These difference spectra have been calculated according to Δm = Aₘₓ₋ₐₓ, where Aₘₓ is the absorbance spectrum at 80 °C and Aₓ is an absorbance spectrum at the temperature x. Two negative bands at 1631 and 1680 cm⁻¹ reflect the disappearance of antiparallel β-pleated sheet structures upon temperature-induced unfolding. The broad band between 1651 and 1680 cm⁻¹ (Fig. 3A) indicates the thermally induced formation of unordered structures. These spectroscopic changes induced by thermal unfolding of RNase
A will be compared quantitatively with the spectroscopic effects observed during the oxidation of RNase A by hydrogen peroxide. For quantification of the fraction of unfolded protein, the absorbance/temperature dependence of the most prominent amide I contour at 1631 cm\(^{-1}\) (low frequency \(\beta\)-band) was analyzed (see Fig. 4). Obviously, three main phases of this absorbance/temperature plot can be observed: a linear low temperature region (below 53 °C), a sigmoidal transition region (53–72 °C), and a second linear post-transitional high temperature region (below 53 °C), a sigmoidal transition region (above 72 °C). The midpoint (inflection point) of the protein melting curve \(T_m\) at which 50% of RNase A is supposed to be in the unfolded state, was determined by curve fitting to be 64.5 °C, in accordance with literature data (Backmann et al. (29): 63 °C). The absorbance change of the 1631 cm\(^{-1}\) band (antiparallel \(\beta\)-pleated sheet structure) is concentration-independent. Quantitative estimations of other structure-sensitive IR bands (e.g. the tyrosine ring vibration band at 1515 cm\(^{-1}\)) were carried out in the same way.

The spectroscopic features of the temperature-induced reversible unfolding and the partial denaturation of RNase A induced by \(\text{H}_2\text{O}_2\) are illustrated in Fig. 5. The upper curve of Fig. 5 (curve a) shows a second derivative spectrum of the native protein RNase A in the amide I' and the amino acid side chain absorption region (after complete H/D exchange, \(T = 30°C\)). The amide II band (near 1550 cm\(^{-1}\)), typical for \(^1\)H protein solutions, can be found in \(^2\)H solutions near 1450 cm\(^{-1}\) (amide II'-band, not shown). Spectrum b was acquired near the melting point of the protein \(T_m = 64.5\) °C, where ~50% of the protein is unfolded. In contrast, spectrum c was recorded after 48 h of incubation of a solution containing initially 4 \(\mu\)mol of \(\text{H}_2\text{O}_2/\text{mg of RNase A}. Spectrum d was obtained at a temperature of 80 °C, where RNase A is expected to exist in a completely unfolded state. The second derivative spectra a–d were normalized utilizing the tyrosine ring vibration band at 1515 cm\(^{-1}\) as an internal standard. A comparison of spectra b (temperature induced unfolding) and c (oxidation) indicates a high degree of conformity of the IR spectroscopic changes, particularly in the secondary structure-sensitive amide I' region.

The temperature behavior of the most prominent IR bands (amide I contour at 1631 cm\(^{-1}\) and the amino acid side chain absorptions) is indicated. Spectrum b was acquired near the melting point of the protein \(T_m = 64.5\) °C, where ~50% of the protein is unfolded. In contrast, spectrum c was recorded after 48 h of incubation of a solution containing initially 4 \(\mu\)mol of \(\text{H}_2\text{O}_2/\text{mg of RNase A}. Spectrum d was obtained at a temperature of 80 °C, where RNase A is expected to exist in a completely unfolded state. The second derivative spectra a–d were normalized utilizing the tyrosine ring vibration band at 1515 cm\(^{-1}\) as an internal standard. A comparison of spectra b (temperature induced unfolding) and c (oxidation) indicates a high degree of conformity of the IR spectroscopic changes, particularly in the secondary structure-sensitive amide I' region.
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Fig. 6. Concentration and time dependence of hydrogen peroxide-induced alterations of RNase A structure. A shows FT-IR difference spectra of RNase A, incubated with varying H2O2 concentrations (T = 30 °C, Δt = 48 h). Difference spectra were calculated according to Equation 1. Negative bands indicate disappearance of structure elements upon RNase A oxidation. H2O2/RNase A concentration ratios were as follows: 0 (a, control), 0.5 (b), 1.0 (c), 2.0 (d), and 4.0 μmol/mg of protein (e). B and C illustrate the time dependence of the spectral changes of RNase A at 4 μmol of H2O2/mg of protein concentration (pH 7.1, Δt max = 48 h). C, illustration of the spectral changes below 1500 cm⁻¹. A small positive peak in the difference spectra at 1047 cm⁻¹ (R2)-S=O stretching vibration?) is indicated by an arrow. The amplitude of this difference peak is comparable with the intensity changes of the tyrosine band at 1515 cm⁻¹ (also marked). AU, absorbance units.

The difference spectra, given in Figs. 3B and 6B, also exhibit significant variations in the spectral region from 1620 to 1580 cm⁻¹. While the difference spectra obtained upon reversible thermal unfolding of RNase A display no significant changes in this spectral region (Fig. 3B), at least one positive band at 1594 cm⁻¹ is found for the RNase A oxidation measurement series (Fig. 6B). This band is assigned in the literature to an asymmetric carboxylate stretching vibration (ν(C=O) asym). The generation of additional carboxylate groups during protein oxidation may be interpreted as a result of disruption of covalent bonds of amino acid side chains. To some extent, however, these spectral features may overlap with a so-called β-aggregation band near 1615 cm⁻¹. The formation of protein aggregates is widely observed for many proteins (48–51). A more detailed examination of difference spectra in Fig. 6B reveals the simultaneous appearance of an additional shoulder at 1714 cm⁻¹ in the positive band contour around 1695 cm⁻¹, which is not present in the difference spectra of the unfolding experiments (Fig. 3B). Carbonyl stretching bands can be expected around this wave number. Some authors reported the generation of carbonyl groups during the oxidation of amino acid side chains producing carbonyl derivatives (1, 52).

Fig. 6C shows the difference spectra of Fig. 6B below 1500 cm⁻¹. These difference spectra display a small positive peak at 1047 cm⁻¹ (R2)-S=O stretching vibration), which is indicated by an arrow. The amplitude of this difference peak is compa-
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The ratio of H₂O₂/RNase A concentrations.

Parallel difference spectroscopy using the low frequency band of antiparallel β-pleated sheets at 1631 cm⁻¹ as a secondary structure-sensitive monitor (A) or a local infrared marker (absorbance of the tyrosine ring vibration band near 1515 cm⁻¹) (B). See inset for the ratio of H₂O₂/RNase A concentrations.

Fig. 7. Time and concentration dependence of hydrogen peroxide-induced structural changes of RNase A as seen by FT-IR difference spectroscopy using the low frequency band of antiparallel β-pleated sheets at 1631 cm⁻¹ (Fig. 7A) and the absorbance values of the tyrosine band at 1515 cm⁻¹ (Fig. 7B). To estimate quantitatively the changes of distinct structural elements as a function of H₂O₂ treatment, the following model was used. From the temperature gradient measurements, the value of the parameter (ΔA/A)₃⁰°C is known. In the example of Fig. 4, this ratio indicates the maximal absorbance change of the low frequency antiparallel β-pleated sheet band at 1631 cm⁻¹, which is a concentration-independent ratio describing complete unfolding of the protein. According to Equation 2, this ratio can be used to estimate the percentage of hydrogen peroxide induced structural changes of RNase A.

\[
\text{Loss of structure elements (t)} = \frac{P_0 - P_1}{P_0 - P_{unfolded}} \times 100\% \quad \text{(Eq. 2)}
\]

The denominator of this equation was calculated from spectral parameters of the temperature profile measurements, and the numerator was calculated from parameters of the hydrogen peroxide experiments. \(P_0\) and \(P_1\) are any structure-sensitive spectroscopic parameter like the absorbance at a given frequency, band frequency, or the half-width at \(t = 0\) or at a given time \(t\), respectively. We used Equation 2 to compare semiquantitatively the structural changes of RNase A oxidation with the structural changes detected during temperature-induced unfolding. This approach allowed us to analyze structural changes in RNase A using the infrared marker bands from the amino acid side chains or secondary structure-sensitive components of the amide I band.

Fig. 7 shows the time and concentration dependence of the absorbances of two distinct spectral marker bands: the low frequency antiparallel β-pleated sheet band at 1631 cm⁻¹, and the tyrosine band at 1515 cm⁻¹. Generally, the higher the concentration of hydrogen peroxide and the longer the incubation time, the larger were the structural alterations detected by FT-IR spectroscopy. The plots of Fig. 7, A and B, and the quantitative results of Table I derived from these plots suggest that substantial differences exist between various secondary structure-sensitive marker bands (amide I band, absorbances at 1680, 1651, and 1631 cm⁻¹) and IR bands of defined amino acid residues (absorbance of the tyrosine and aspartate band at 1515 and 1584 cm⁻¹). While the direction and the time-dependent changes in the amide I region indicate an “unfolding-like” behavior of the protein as a result of H₂O₂ treatment, the information derived from the amino acid side chain bands cannot be explained simply on the basis of protein unfolding (see ordinate values of Fig. 7B). These interesting findings will be discussed below.

Oxidation of RNase A by Hydrogen Peroxide (120-h Incubation)—Armed with the knowledge that the oxidative damage of RNase A may be accompanied by conformational and covalent modifications, we undertook a series of experiments to detect subtle spectral changes, especially of the amino acid side chain absorptions. Those measurements required a very high signal/noise ratio and instrument stability. Therefore, the sampling time for each individual spectrum of these experiments was increased to 20 min (2200 scans), and the time of data collection was increased to 120 h. The experiments shown in Fig. 7 (48-h duration) were carried out at a temperature of 30°C. The protein concentration and the composition of the buffer solutions were not changed. To analyze the smallest spectroscopic effects detectable and to minimize unavoidable baseline shifts (a common problem of long time measurements), the spectroscopic parameters were calculated exclusively from second derivative spectra (see Fig. 8). High signal/noise ratio (see Fig. 8, B or C), stability of the experimental setup over 5 days (temperature, water vapor content, etc.), and state-of-the-art data evaluation were essential prerequisites to be able to obtain information from these types of experiments.

The interpretation of band parameters derived from second derivative spectra is not as straightforward as for data obtained from the original absorbance spectra. The calculation of d(A)/d(ΔT) (values from a simulated Lorentzian band) yields a negative band where the position of the minimum of the second derivative band coincides with the frequency value of the maximum of the original Lorentzian, while the ordinate value at the minimum depends on the half-width and the absorbance value of the Lorentzian band. Therefore, one can utilize second derivative intensities for comparative purposes in the same way as absorbances from original spectra, provided that the half-widths of the bands are constant and do not depend on temperature or other experimental parameters. For the determination of band frequency, second derivatives are even more precise than the original absorbance spectra.

In Fig. 8, the low frequency β-pleated sheet band absorbance at 1631 cm⁻¹ (Fig. 8, A and D) and the absorbance and the frequency values of the tyrosine band at 1515 cm⁻¹ (Fig. 8, B, C, E, and F) were evaluated from second derivative spectra. While the curves in the left column of Fig. 8 (A–C) display the time dependence of the structural alterations of RNase A induced by hydrogen peroxide, the plots of the right column (D–F) show the corresponding changes during temperature-induced unfolding of the protein. The curves (I) of the left panels display the fraction of structural change during RNase A oxidation at 1 μmol of H₂O₂/mg of protein, while the corresponding results of the control experiment without H₂O₂ are given by curves (II). The protein melting curves of Fig. 8, D and E, were corrected for linear temperature effects and normalized according to a method proposed by Heyn (53). The calculation of folded/unfolded protein fractions in the hydrogen peroxide measurement
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TABLE I
Quantitative estimations of changes upon H₂O₂ treatment of RNase A marker bands (Δt = 48 h)

| Concentration of H₂O₂ | Temperature run |
|-----------------------|-----------------|
| 0 min     | 5 min | 10 min | 20 min | 40 min |
| 0% | 3% | 13% | 34% | 42% | 53% | 100%
| 1% | 2% | 8% | 12% | 40% | 54% | 100%
| 3% | 3% | 40% | 72% | 103% | 193% | 100%
| 5% | 1% | 7% | 50% | 57% | 100% |
| 7% | 0% | 60% | 173% | 205% | 100% |

| Oxidation of RNase A by H₂O₂ (time dependence, isoformal) | Temperature induced unfolding of RNase A |
|----------------------------------------------------------|---------------------------------------|
| ![Graph](image1)                                         | ![Graph](image2)                      |

**FIG. 8.** A comparison of spectral changes induced by temperature increase of RNase A solutions (unfolding) and hydrogen peroxide-induced structural alterations of RNase A. All spectroscopic parameters of this figure were calculated from second derivative spectra. Left, time dependence of band parameters obtained on a hydrogen peroxide/RNase A incubation experiment (I) and the respective control experiment (II). The relation of intensity values at a given time t and t = 0 (A and B) or frequency values (C) were calculated and plotted as a function of time. Curves A were obtained, using the absorbance information at 1631 cm⁻¹ (low frequency β-sheet band), while B and C display the time dependence of the absorbance or frequency parameters of the tyrosine band at 1515 cm⁻¹, respectively. Right, temperature profile measurements of RNase A. D and E display the normalized second derivative/temperature dependence at 1631 and 1515 cm⁻¹, respectively, while F shows the frequency/temperature dependence of the tyrosine band. A comparison of the curves in the left and right rows indicates that hydrogen peroxide-induced structural alterations of RNase A are to some extent similar to spectral changes during the unfolding of the protein. However, the structural information derived particularly from infrared marker bands of the amino acid side chains makes it evident that additional “local” events (e.g., oxidation of aromatic residues) may take place (see “Results”).

A series was carried out using Equation 2, where the spectral parameters P were obtained from second derivative spectra.

**Curve I** in Fig. 8A indicates a significant decrease of the absorbances of the low frequency β-band intensity at 1631 cm⁻¹ in accordance with the measurement series of Figs. 5–7. In general, the magnitude and the direction of the time-dependent changes detected in the amide I region coincide with those observed during thermal unfolding of RNase A. In contrast, the spectral changes of the tyrosine band at 1515 cm⁻¹ (induced by hydrogen peroxide or by temperature variation) have an opposite direction. As indicated by the FT-IR difference spectra of Fig. 6, A–C, the intensity of the tyrosine band is decreasing as a function of hydrogen peroxide concentration and incubation time, while it is increasing with temperature-induced unfolding of the protein. Consequently, the ordinate values of Fig. 8B are negative (see also Fig. 7B). Specifically, the absorbance changes in the amide I region indicate a decrease of about 70% of secondary structure elements after 120 h of incubation time at 1 μmol of H₂O₂/mg of protein, while the corresponding tyrosine band absorbances changes are comparable in the magnitude but opposed in direction. Interestingly, the absorbance time-dependence of the “aspartate band” at 1584 cm⁻¹ exhibits much larger changes in the oxidation experiment compared with the effects of the unfolding measurements (cf. Table I). But in contrast to the behavior of the tyrosine band, the intensity of the aspartate band at 1584 cm⁻¹ is diminishing upon unfolding of RNase A as well as upon oxidation. Thus, relative absorbance changes calculated by Equation 2 were found to be positive for the aspartate band at 1584 cm⁻¹ and negative for the tyrosine band at 1515 cm⁻¹ (Table I).

In analogy to the analysis of the band intensity of the β-structure band at 1631 cm⁻¹, the frequency analysis of the tyrosine ring vibration band at 1515 cm⁻¹ indicates similarity between temperature (Fig. 8F) and hydrogen peroxide (Fig. 8C)-induced structural alterations of RNase A. The temperature-induced unfolding is accompanied by a tyrosine “peak shift” to higher wave numbers by about Δδ₉⁰°C ~ 0.4 cm⁻¹. A similar effect was observed for the 120-h H₂O₂ incubation experiment, where a tyrosine peak shift of about 0.35 cm⁻¹ indicates a global unfolding of about 70% of the RNase A molecules. Interestingly, this value is close to that determined from the high frequency β-pleated sheet band at 1680 cm⁻¹ (not shown) and from the low frequency β-pleated sheet band at 1631 cm⁻¹ (cf. Fig. 8A). Presumably, the intensity of the IR marker bands such as the high and the low frequency β-pleated sheet band at 1680 and 1631 cm⁻¹ and the frequency of the tyrosine band monitor global folding/unfolding events of the protein, while other spectral markers such as the intensities of the tyrosine and the aspartate bands reflect more local consequences of protein oxidation (chemical modifications).

**Time Dependence of the Proteolytic Susceptibility of RNase A**—As demonstrated by Fig. 1, the reaction with hydrogen peroxide up to 4 μmol of H₂O₂/mg of RNase A leads to an increase of proteolytic susceptibility of RNase A. Since it is
known that protein damage is not only a concentration-dependent but also a time-dependent process, we also investigated the time dependence of the proteolytic susceptibility of RNase A. In this study, only the incubation time of RNase A with hydrogen peroxide was varied, while the reaction time of the 20 S proteasome with the oxidized RNase A was kept constant (2 h). In this way, we measured the proteolytic susceptibility of the substrate and not a maximal amount of protein oxidation. As shown in Fig. 9, a time-dependent increase in the proteolytic susceptibility of the RNase A up to 48 h was detected. At all time points tested, a clear dependence of proteolytic rates on the H$_2$O$_2$ concentration was found.

Correlation of Proteolytic Susceptibility and FT-IR Spectroscopic Changes—One aim of this study was to get new insights into the molecular basis of the recognition process of mildly oxidized RNase A by the 20 S proteasome. Assuming that distinct structure-sensitive IR spectroscopic parameters of oxidized RNase A are related to the proteolysis rates, we correlated hydrogen peroxide-induced changes of specific infrared bands with the proteolytic susceptibility of RNase A toward the 20 S proteasome.

Fig. 10 clearly shows that hydrogen peroxide-induced structural reorganization of the protein coincides with increased proteolysis rates of RNase A. For all infrared bands analyzed in this study, i.e. for “global” as well as for “local” parameters, a clear linear dependence between structural changes and the proteolytic susceptibility was detected. Consequently, this approach did not allow the identification of a specific recognition site causing proteasome binding and subsequent protein degradation. However, an improved experimental setup to increase the sensitivity and the time resolution of the experiment may be helpful to address this problem more adequately.

**DISCUSSION**

Covalent modifications of proteins by oxidative agents are thought to play a key role in various physiological and pathological conditions such as inflammation, ischemia reperfusion, or aging (for reviews see Oliver et al. (54) and Stadtman (55)). It has been shown that oxidative modification of proteins can be mediated by a number of different systems including oxidases, ozone, hydrogen peroxide, hypochloride, superoxide, γ-irradiation, and metal-catalyzed oxidation, to mention a few. As a result of protein oxidation, an accumulation of enzymes with partially altered structure and function is observed. These proteins exhibit changes in thermotability (41) and, when mildly oxidized, show an increased susceptibility to degradation by proteasome (1, 2, 5, 6, 15, 18), which is known to be a part of a complex antioxidant repair and removal system. This main intracellular proteolytic system for the degradation of oxidatively damaged proteins exists in at least two forms, the ATP- and ubiquitin-dependent 26 S form and an independent 20 S form. The 20 S proteasome used in this study is a 700-kDa soluble protease complex that is found in the cytosol and the nucleus of mammalian cells (56). Proteolysis of oxidatively damaged proteins by the 20 S form of the proteasome seems to be the major pathway (5, 6, 18). As observed by a number of studies, proteolysis by the 20 S proteasome is most efficient after treatment of the protein with moderate oxidative stress, whereas greater oxidative damage actually leads to decreased proteolytic susceptibility (15, 17, 57). It has been shown that the formation of protein aggregates, by whatever mechanism, can contribute to the decrease of proteolytic rate (41).

However, the molecular basis of the recognition process of mildly oxidized proteins by the proteasome still remains questionable. Experimental evidence for a possible role of increased surface hydrophobicity (16, 20–22), the formation of dityrosines (57), the conversion of methionine to methionine sulfone (21), and the increase of reactive carbonyl content (21) were found. Nevertheless, an *in vitro* study showing a direct relationship between structural changes of a model protein and proteolytic susceptibility to the 20 S proteasome is still lacking. To get new insights into the recognition process of mildly oxidized proteins by the 20 S proteasome, we combined a structure-sensitive spectroscopic technique, FT-IR spectroscopy, with measurements of the proteolytic susceptibility of RNase A toward the 20 S proteasome. We report here experimental evidence for oxidation-induced conformational rearrangements of the secondary structure of the model protein RNase A and, at the same time, for covalent modifications of amino acid side chains such as tyrosine and aspartate residues. These modifications could be correlated to the proteasome-induced proteolysis rate of oxidatively damaged RNase A.
Oxidatively damaged RNase A can be degraded by the 20 S proteasome. This degradation exhibits a typical biphasic dependence; at comparatively low concentrations of \( \text{H}_2\text{O}_2 \), the proteolytic rate of the mildly oxidized protein is increased if the oxidant concentration is increased. At concentrations above 4 \( \mu \text{mol} \) of hydrogen peroxide/mg of protein, an inverse relation is observed (cf. Fig. 1). These findings are in good agreement to literature data for other model systems (6, 15, 58), which were a starting point for the present study.

The remarkably high degree of similarity between the infrared difference patterns, particularly in the amide I region, for oxidation- and temperature-induced unfolding of RNase A prompted us to propose a correlation between hydrogen peroxide-induced protein modifications and protein unfolding. In fact, the similarity of spectral characteristics observed for both processes (cf. spectra b and c in Fig. 5) support the hypothesis that oxidative damage of proteins may result in global unfolding rearrangements of the polypeptide. This is also corroborated by the thermal and denaturant-induced unfolding of RNase A (29). The proposed correlation is supported by two facts. First, a quantitative comparison of RNase A oxidation using several spectroscopic parameters of the amide I region (such as high and low frequency \( \beta \)-band or the band for \( \alpha \)-helical structure) indicate a remarkably high degree of internal consistency (Table 1). Second, even when relatively high concentrations of the oxidants were applied for time periods up to 120 h, the changes of all secondary structure elements of the amide I region were always smaller than the changes observed during the temperature-induced two-state transition of global unfolding. Thus, the results of the protein oxidation experiments suggest that the hydrogen peroxide-induced structural rearrangements of RNase A are processes similar to global unfolding, involving all parts of the protein. Protein backbone fragmentation may be a potential candidate producing unfolded fragments of RNase A. However, the results of mass spectrometry demonstrate that oxidative unfolding of RNase A does not perturb the protein sequence.

The detailed analysis of some amino acid side chain absorption bands indicates distinct discrepancies that cannot be explained on the basis of the simple model of protein unfolding. While the absorbance values of the tyrosine band at 1515 cm\(^{-1}\) are increasing during temperature-induced unfolding of RNase A, the oxidation experiments exhibit a decrease of this band. Furthermore, for the absorption band of the aspartate residues at 1584 cm\(^{-1}\) we found a decrease in intensity twice as large compared with the corresponding changes observed during complete unfolding. From the literature, it is known that side chains of proteins are primary targets of oxidation (e.g., Ref. 57). Therefore, it is possible that some of the tyrosine (and aspartate) side chains of RNase A were oxidized by hydrogen peroxide. Tyrosine is one amino acid residue that can readily undergo oxidation, forming a number of reaction products such as dityrosines (57). RNase A contains six tyrosine residues and, as the calculations of the ASA show, only one of these side chains is initially not accessible by the solvent (Tyr²⁵). The remaining residues revealed relative ASA values between 11 and 65% (Tyr²⁵ and Tyr⁶⁵). Therefore, an oxidative modification of at least some tyrosine residues seems likely.

Unlike the absorbances of the tyrosine band, the time dependence of the tyrosine band frequency confirmed quantitatively the corresponding structural alterations found in the amide I region (see Table 1). It is expected that any covalent modification of the tyrosine ring will change its spectral characteristics considerably. Thus, oxidation of the tyrosine residues will necessarily be followed by a decrease of the tyrosine band intensity at 1515 cm\(^{-1}\) (and accordingly by the appearance of a number of new bands), whereas the frequency of the tyrosine band should remain unchanged. Therefore, we interpret frequency shifts of the tyrosine band during RNase A oxidation as a consequence of changes in the microenvironment, in this case due to the unfolding of the protein.

Previous literature demonstrated (25) that under nonphysiological high concentrations of the oxidant, proteins may aggregate to large and densely packed complexes that are not accessible to proteases. Aggregates can be either formed by covalent cross-linking (dityrosines; Ref. 57) or by intermolecular hydrophobic interactions (intramolecular \( \beta \)-sheets; Ref. 20). According to these well established facts, the decrease of proteasome degradation activity at high \( \text{H}_2\text{O}_2/\text{protein} \) concentration ratios (cf. Fig. 1) was correlated to the formation of aggregates of this protein. Previous IR studies have shown (48, 50, 51) that densely packed intermolecular \( \beta \)-aggregates give rise to highly characteristic infrared bands. Particularly, a so-called \( \beta \)-aggregation band at 1615 cm\(^{-1}\) is known to be typical for the presence of these aggregates. The FT-IR measurements of the present study, however, were carried out at comparably low \( \text{H}_2\text{O}_2/\text{protein} \) concentration ratios, i.e. when protein aggregation is supposed to play only an inferior role. It cannot be ruled out completely that the difference spectra shown in Fig. 6B show small signs of an aggregation band. However, the presence or absence of a minor \( \beta \)-aggregation band seems to be irrelevant for the interpretation of the data of the present study.

Oxidative modification of amino acid side chains can severely reduce the conformational stability of proteins. Stadtman and colleagues (52) used an example of two-model proteins to demonstrate that during exposure of amino acids to ozone. Primarily methionine and aromatic amino acid residues were oxidized in the order Met > Trp > Tyr ∼ His > Phe (52). Hence, methionine residues appear to be the primary targets of oxidation. It was furthermore proposed that methionine residues constitute an effective antioxidant on the surface of proteins (14). Since the oxidation product of methionine, methionine sulfoxide, can be recycled by a catalytic system, methionine residues may act as oxidant scavengers. Interestingly, for many proteins it was demonstrated that protein conformation is little affected if solvent-accessible methionines on the protein surface are oxidized (14, 59–61). These studies support the hypothesis that surface exposed methionines generally preserve the biological function of the protein.

The S-C stretching vibration of methionines (R-CH₂-S-CH₃) can be expected in the spectral region of 730–570 cm\(^{-1}\) (62). The experimental setup used in this study (CaF₂-windows) did not allow us to monitor IR-spectroscopic changes in this low frequency region. Nevertheless, the absorption band arising from the S=O stretching vibration of methionine sulfoxide (R₂=S=O) is known to occur between 1060 and 1015 cm\(^{-1}\) (62). In fact, at 1047 cm\(^{-1}\) a small and narrow positive band (cf. Fig. 6C) was observed for the RNase A oxidation experiments, while the control measurements did not indicate any additional peak at these wave numbers. In RNase A, the four methionine residues are poorly accessible by the oxidant. For the side chain of Met²⁹, an ASA value of 18% was calculated (Met¹³, 7%; Met³⁰, 0%; Met⁷⁹, 9%). However, we demonstrated that large scale disorganization of the protein conformation occurs upon \( \text{H}_2\text{O}_2 \) treatment, which implies that Met residues could have been oxidized that were initially shielded.

The other aromatic amino acid side chains of RNase A such as Phe (there is no Trp in RNase A) are also poorly accessible. For three Phe side chains, ASA values between 0 and 8% were calculated. On the other hand, the three His residues of RNase A, which are also known to undergo oxidative modification,
exhibit ASA values of >35%. Therefore, we think that in native RNase A, the primary targets of oxidation are tyrosine and histidine residues. This could be shown convincingly by FT-IR difference spectroscopy at least for tyrosine residues.

A summarizing description of possible conformational and covalent modifications of proteins during oxidative damage is given in Fig. 11. Areas A–D of Fig. 11 schematically illustrate how the concentration of the different RNase A fractions may vary if the concentration of the oxidant is increased. Initially, at comparably low oxidant concentration, some of the solvent-accessible amino acid residues such as histidine, methionine, or aromatic amino acid residues are modified (Fig. 11B). The FT-IR spectroscopic results of this study clearly indicate that tyrosine and aspartate amino acid residues are involved. Possibly, the more side chains are oxidized by hydrogen peroxide, the more the conformational stability of the protein is reduced and the higher the probability of local or even global unfolding (Fig. 11C). Unfolding causes the exposure of initially hydrophobic groups to the solvent and accordingly to the oxidant. Thus, residues that were initially not exposed to the solvent were made accessible to oxidation by hydrogen peroxide and other secondary oxidants. As a result, the protein surface hydrophobicity, potentially one of the recognition signals for proteasome binding, is increased. However, with increasing concentrations of the unfolded protein, the probability of intermolecular formation of protein aggregates that are not accessible to proteasome digestion will increase as well (Fig. 11D). Therefore, at nonphysiologically high concentrations of the oxidants, the proteasome turnover is not further enhanced but will be reduced instead. This situation is reflected in Fig. 11, where a difference curve between the unfolded fraction of RNase A (C) and aggregated forms of the protein (D) is displayed. Interestingly, the slope of this difference curve matches the main features of the proteasome turnover plot of Fig. 1. However, it cannot be completely ruled out that products of the oxidative modification of amino acid side chains, such as methionine sulfoxide, play a significant role in the proteasome recognition process as well. Yet we believe that hydrophobicity is the key signal for the 20S proteasome because of the initially low accessibility of the methionine residues. However, additional experimental efforts are necessary to answer these questions. FT-IR difference spectroscopy is one of the potentially techniques for detecting oxidant-induced changes in protein structure due to its very high sensitivity and specificity. Further increase of the specificity of the method may be achieved by the use of isotopic labeled compounds e.g. of methionines marked by $^{13}$C and $^{15}$N and/or the substitution of specific amino side residues.

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