RAMAN SPECTRA OF *N*-FORMYLKYNURENINE DERIVATIVES OF LYSOZYME PRODUCED BY OZONE OXIDATION

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Received 23 July 1979

1. Introduction

It is well established that in photosensitized reactions of proteins as well as in processes induced by air pollutants the oxidation of aromatic amino acid sidechains plays an important role. As a consequence changes of the three-dimensional structure are frequently observed.

We have pointed out [1] that Raman spectroscopy is an excellent method for studying simultaneously chemical modifications of aromatic amino acid sidechains and conformational changes. We reported results on lysozyme derivatives formed from the native enzyme by oxidation with *N*-bromosuccinimide. By this agent mainly tryptophan sidechains were converted to oxindole alanine residues.

Here it is shown that Raman spectroscopy is also well suited for studying problems stated above. We have investigated the oxidation of lysozyme by ozone which is known to be an important constituent of photochemical smog. Its action on proteins mainly results in the formation of *N*-formylkynurenine from tryptophan residues. In addition depending on the experimental conditions [2–5] further amino acid side chains are modified. The choice of lysozyme enables us to compare our Raman spectroscopic results with investigations using other methods [2–5].

2. Experimental

Hen egg-white lysozyme (EC 3.2.1.17) was purchased from Boehringer-Mannheim. It was oxidized in aqueous solutions (1%) by a slow stream of ozone generated by silent electric discharge in oxygen (Gallenkamp ozonizer GE 150). The ozone concentration (0.03% in O_2) was determined by titration of iodine liberated during the reaction of the oxidizing agent with KI.

All 6 tryptophan residues were destroyed when the stream of ozone was passed through a lysozyme solution for ~17 h. The product was purified twice by Sephadex G-25 chromatography. Three components could be detected. The main fraction was lyophilized and investigated Raman spectroscopically (OL VI).

By stopping the reaction after shorter ozonization times products with various numbers of oxidized tryptophan residues were isolated. Two species were investigated after purifying them as above. In OL II and OL III about 2 and 3 Trp residues were oxidized, respectively.

The content of tryptophan in the oxidized lysozyme species was measured by two methods, from the absorption spectra by the procedure in [6] and from the decrease of the indole ring vibrations at 760 and 1011 cm^{-1} in the Raman spectra. We have shown in [1] that the intensity of these bands decreases approximately proportional to the number of the oxidized tryptophan residues.

The following compounds were commercial products: L-cysteic acid (Sigma); *N*-formylkynurenine (Calbiochem).

Details of the Raman spectroscopic equipment were described in [1].

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3. Results and discussion

3.1. *Tryptophan and* N-*formylkynurenine vibrations*

In the case of OL VI the Raman spectrum (fig.1b) shows unequivocally that all Trp residues have been destroyed. This conclusion can be drawn from the complete vanishing of the indole ring vibrations [7] at 760 and 1011 cm^{-1} (cf. fig.1a) and the disappearing of the stretching vibrations of the aromatic system of tryptophan in the range of 1550–1620 cm^{-1}. Instead, the intensity of the band at 1604 cm^{-1}, which can be assigned to the benzene system of *N*-formylkynurenine (fig.3) increases remarkably.

A weakening of tryptophan bands can also be observed in the case of the other lysozyme oxidation products (fig.2). In all oxidized species a new band arises at 1052 cm^{-1}, which can also be detected in the Raman spectrum of *N*-formylkynurenine (fig.3). In the case of the stronger oxidized derivative OL VI this vibration overlaps with a second peak resulting in a broad band with a maximum at 1042 cm^{-1} (fig.1b). The origin of the additional peak will be discussed later.

3.2. *Tyrosine vibrations*

The spectra of all oxidized lysozyme species reveal the characteristic tyrosine vibrations at 643 cm^{-1} and the Fermi resonance doublet at ~830 and 855 cm^{-1}. In OL VI (fig.1b) these bands are noticeably weakened, which indicates the oxidation of tyrosine residues too. By comparing the Fermi resonance doublet of OL III (fig.2) and of the native protein (fig.1a) a striking change of the intensity ratio I_{855}/I_{830} can be observed. This ratio is found to be sensitive to the strength of the hydrogen bonds of the tyrosine hydroxyl groups in proteins [9]. Therefore it can be concluded that OL III and native lysozyme differ with respect to their three-dimensional structure. Similar conclusions have been drawn from CD measurements [4].

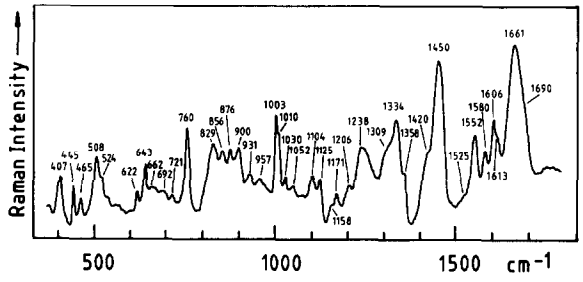

Fig.2. Raman spectrum of a lysozyme derivative with three oxidized Trp residues (OL III, lyophilized); excitation, 647 nm.

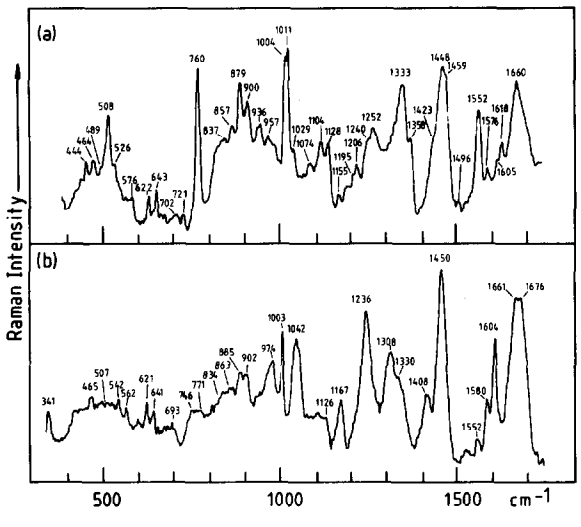

Fig.1. Raman spectra (a) of native lysozyme (crystals) and (b) of a lysozyme derivative with six oxidized Trp residues (OL VI, lyophilized); excitation, 647 nm.

3.3. *NHCO-vibrations*

Especially in the higher oxidized lysozyme derivatives a low frequency shift in the amide III range (1230–1280 cm^{-1}) and in the amide I region is clearly seen. In OL VI two maxima in the amide I band at 1661 and 1675 cm^{-1} can be detected (fig.1b).

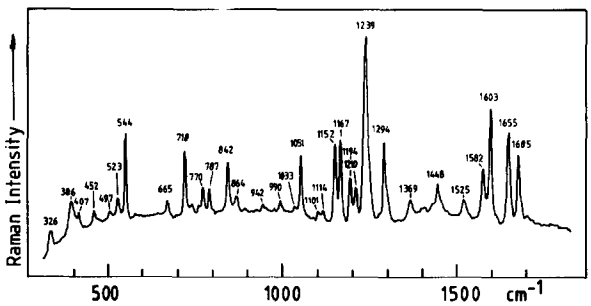

Fig.3. Raman spectrum of *N*-formylkynurenine (crystal powder); excitation, 647 nm.

Normally shifts in the amide I and amide III range indicate conformational changes of the peptide backbone of proteins. However, in the spectra of the oxidation products containing *N*-formylkynurenine side chains an interpretation of these shifts with respect to conformational changes is impossible. The two strong bands of *N*-formylkynurenine at 1239 and 1685 cm^{-1} (fig.3), which can be assigned to vibrations of the *N*-formyl group, superimpose the amide regions. These vibrations are probably primarily responsible for the observed shifts.

3.4. *Disulfide vibrations*

In the lower oxidized species no change could be observed in the disulfide range (508 cm^{-1}). This means that no *S–S* bridges were attacked by O_3 and the CS–SC dihedral angles are unchanged compared with the native protein [8]. On the other hand, in the highest oxidized derivative OL VI the S–S vibrations are no longer detectable (fig.1b). Thus it can be concluded that in OL VI all disulfide bridges were destroyed. Instead a strong band appears at 1042 cm^{-1} which overlaps with the *N*-formylkynurenine peak at 1051 cm^{-1}.

The product of the cystine oxidation is cysteic acid [3,5]. This compound shows its strongest band at 1036 cm^{-1}. As to be expected the same band can also be observed when a solution of cystine is oxidized by ozone. Therefore we assign the broad band at 1042 cm^{-1} (fig.1b), which could only be detected with the lysozyme derivative OL VI containing oxidized disulfide groups, to a superposition of the *N*-formylkynurenine peak at 1051 cm^{-1} and the band of cysteic acid at 1036 cm^{-1}.

Acknowledgements

The authors wish to thank the Stiftung Volkswagenwerk, the Deutsche Forschungsgemeinschaft, the Erwin-Riesch-Stiftung and the Vereinigung von Freunden und Förderern der Johann Wolfgang Goethe-Universität Frankfurt a.M. for the generous support of their work and Mrs U. Dietzel for technical assistance.

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