MÖSSBAUER SPECTRA OF I^{129} IN TOBACCO MOSAIC VIRUS (*)

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Résumé. — A l'aide des isotopes I^{129} fixés au virus mosaïque de tabac, on peut effectuer des études par spectroscopie Mössbauer qui fournissent des informations sur la structure quaternaire de cette molécule.

En ce qui concerne leurs liaisons chimiques, des mesures de la constante d'interaction quadrupolaire ont été réalisées aux amino-acides tyrosine 139 et cystéine 27 contenus dans le virus mosaïque de tabac. Le paramètre de ces expériences est la valeur pH.

Les résultats prouvent l'existence d'une liaison d'hydrogène dans le virus provenant du groupe hydroxyle de la tyrosine 139 et excluent la présence d'une liaison de cystine s'appuyant sur la cystéine 27.

Abstract. — The observation of the electric quadrupole coupling constant of I^{129} in tobacco mosaic virus provides data related to the quaternary structure of the molecule.

To obtain information on their chemical bonds the amino acids tyrosine 139 and cysteine 27 in tobacco mosaic virus were iodinated and the Mössbauer spectra of the samples were measured at various pH-values.

The results prove the existence of a hydrogen bond involving the hydroxyl group of tyrosine 139, and exclude the formation of a cystine bridge at cysteine 27 in tobacco mosaic virus.

1. Proteins are heterogeneous polymers consisting of 100 to 500 amino acid residues, and processing various catalytic and structural functions. About twenty different amino acids can occur. The three-dimensional structure of the protein is determined and stabilized by non-covalent interactions in addition to the covalent bonds. The most specific of the non-covalent interactions are hydrogen bonds. In the physiological functions of proteins the making and breaking of hydrogen bonds is often of importance (e. g. haemoglobin, [1]). However, a typical protein contains hundreds of hydrogen bonds, thus often making it difficult to obtain specific information about a functionally significant hydrogen bond.

The use of NMR spectroscopy has gone some way to dealing with this problem, but unfortunately a large class of interesting biological macromolecules have such high molecular weights that the NMR resonance lines are very broad and unmeasurable. Therefore, there is considerable biochemical interest in other spectroscopic methods, particularly those whereby specific reporter groups may be introduced into the molecule, and which may be used to observe the state of ionisation or a configurational change in the

direct environment of the reporter. For example, Mössbauer spectroscopy has been applied to determine the electronic configuration of the iron atom in haemoglobin. However, when metals bind to proteins they usually do so by forming coordination complexes; except in special cases the binding of metals to proteins is not highly specific so that they are not particularly suitable as reporters. Among the other possible Mössbauer nuclides iodine offers many advantages. For instance, iodine can be made to react specifically and covalently with proteins, or may be introduced into enzyme substrate analogues, and can be used as a specific reporter atom.

In this paper we report an application of the Mössbauer effect to the problem of determining the state of coionization of a side chain involved in a stabilizing hydrogen bond in tobacco mosaic virus (TMV). We have measured the quadrupole splitting of the nuclear levels, and the isomeric shift, which are both sensitive to the electronic configuration near the probe nuclei of the Mössbauer isotope I^{129} .

Iodine occurs naturally in some proteins or may be covalently and specifically attached to the protein or substrate. It offers two isotopes which are suitable for Mössbauer experiments: I^{127} and I^{129} . For the measurements reported here the 27.8 keV transition in I^{129} has been chosen. In comparison to I^{127} it exhibits a

(*) Work supported by research grants of the Deutsche Forschungsgemeinschaft.

line width smaller by a factor of four, so that completely resolved Mössbauer lines can be observed. The excited level in question is populated by an isomeric γ transition in $(33.6 \text{ d}) \text{ Te}^{129\text{m}}$ and a succeeding β decay. The absorber nuclei I^{129} have a life time of $1.6 \times 10^7 \text{ y}$.

Tobacco mosaic virus was selected as an example of a complex protein structure because of several advantageous features :

- the structure contains about 2 000 identical protein subunits
- the structure of the virus has been investigated thoroughly by X-ray methods [2]
- the polypeptide chain of TMV protein contains the amino acids tyrosine and cysteine, of which tyrosine 139 and cysteine 27 can be iodinated in a well defined way
- TMV is a stable system which is easy to prepare.

Free di-iodo-tyrosine was initially investigated at different pH-values, and it was demonstrated that the Mössbauer parameters are sensitive to the change from the protonated to the non-protonated state. Subsequent spectra for di-iodo-tyrosine in TMV deviate from those taken for free tyrosine at the same pH-values. This change is interpreted in terms of the special conditions of tyrosine 139 in TMV.

In TMV it is possible to substitute the hydrogen in the cysteine by iodine, resulting in a stable S-I bond. The Mössbauer spectra taken from TMV demonstrate the presence of this unusual S-I bond.

In the following chapter we report on the experimental procedure. The results are discussed in chapter 3.

2. Experimental. — The experimental arrangement used was the standard Mössbauer transmission geometry. The source was attached to an electromechanical drive-system working in constant acceleration mode. The data were stored in a multichannel analyzer. The velocity scale was calibrated with a laser interferometer. A $\text{ZnTe}^{129\text{m}}$ single-line source was used. Both source and absorber were maintained at a temperature of 4.2 K during the measurements.

The 27.8 keV γ -rays were detected with a $4 \text{ cm } \varnothing \times 0.1 \text{ cm}$ NaI(Tl) scintillator. The activity of the $\text{Xe}^{129} \text{ K}$ X-rays emitted from the long-lived radioactive absorber was reduced by a Sn critical absorber of 70 mg/cm^2 .

The 3,5-di-iodo-tyrosine absorbers (Fig. 1) with I^{129} were prepared using a modification of the method of Barnes *et al.* [3]. The three absorbers, each $8 \text{ mgI}^{129}/\text{cm}^2$, were

- 3,5-di-iodo-tyrosine (solution pH 2), labelled T 1.
- 3,5-di-iodo-tyrosine (solution pH 10), labelled T 2.
- 3,5-di-iodo-tyrosine (crystallized), labelled T 3.

The pH-values of the solutions were chosen to ensure the protonated and the non-protonated states of the hydroxyl group of the tyrosine.

FIG. 1. — 3,5-di-iodo-tyrosine.

TMV is built up of ribonucleic acid (RNA) and a protein coat. The molecular weight is about 40 million. The protein part consists of about 2,200 identical subunits each with a molecular weight of 17,800. The amino acid sequence of the protein has been determined [4]. Each subunit contains four tyrosine residues and one cysteine. TMV is a favourable case : only tyrosine 139 reacts with iodine at room temperature ; the sulfhydryl group of cysteine 27 can be blocked by methyl mercury nitrate (MMN) (Fig. 2) [5]. However,

FIG. 2. — Cysteine (reaction with iodine ; reaction with methyl mercury nitrate).

it is also possible to iodinate both amino acids, tyrosine 139 and cysteine 27. To prepare iodinated TMV we added a solution of I_2^{129} in NaI^{129} dropwise. For iodination of tyrosine 139 alone we added two equivalents of iodine per subunit of the virus which had previously been reacted with methyl mercury nitrate. For iodination of tyrosine and cysteine three equivalents were added.

The following TMV absorbers were prepared :

- TMV, tyrosine 139 iodinated, pH 3, labelled V 1.
- TMV, tyrosine 139 iodinated, pH 8, labelled V 2.
- TMV, tyrosine 139 and cysteine 27 iodinated, pH 8, labelled V 3.

The Mössbauer spectra of the absorbers exhibit well-resolved quadrupole splittings as shown in figures 3, 4 and 5. The smooth curves drawn in these figures were obtained by a least squares fit of the theoretical quadrupole spectra to the data. The background, intensity, quadrupole coupling constant ($e Q_{gr} V_{zz}$) asymmetry parameter (η), isomeric shift relative to the Zn Te

FIG. 3. — Di-iodo-tyrosine, pH 10, with an I^- impurity near zero velocity.

FIG. 4. — TMV, tyrosine 139 iodinated, pH 8.

FIG. 5. — TMV, tyrosine 139 and cysteine 27 iodinated, pH 8.

source (δ), full width at half maximum (Γ), and recoilless absorption anisotropy (χ), were the parameters varied to obtain the minimum chi-square. The asymmetry parameter is incorporated into the fitting program using the approximation method of Shenoy and Dunlap [6].

The results for the different absorbers are given in table I.

For comparison with data thus obtained, the results of two previously reported measurements have been included in the table. The values listed for DIT are to be compared with those for the crystallized di-iodo-tyrosine, RSI refers to a sample with a S-I bond similar to that observed for cysteine in TMV.

Particularly important parameters in table I for the different absorbers are the electric quadrupole splitting $eQ_{gr}V_{zz}$ and the isomeric shift δ .

3. Discussion. — The data for free tyrosine molecules at different pH-values were obtained in order to elucidate the influence of various chemical bonds on the parameters in question. Depending on the pH of the solution the 3,5-di-iodo-1-tyrosine possesses a hydroxyl group which is either protonated or non-protonated. Changes with respect to the carboxyl or amino group of the molecule are not taken into consideration since these groups are spatially well removed from the active Mössbauer atom.

The pH-value for which both states are equally available in the solution is the pK-value of the hydroxyl group under consideration. The pK-value can be easily determined for the tyrosine molecule by physical means. For 3,5-di-iodo-tyrosine the pK-value is 7 [7]. There is a clear relation between the different configurations and the pH-values. At pH 2 the protonated state predominates in the solution by a factor 10^5 , for pH 10 the non-protonated state by a factor 10^3 . Thus the Mössbauer parameters for quadrupole splitting and isomeric shift obtained for the absorbers T 1 (pH 2) and T 2 (pH 10) are characteristic for the two extreme states of the molecule.

The crystallized tyrosine absorber T 3 and absorber T 1 (pH 2) provide the same Mössbauer parameters.

TABLE I

Results of the Mössbauer measurements

	$-eQ_{gr}V_{zz}$ [MHz]	δ [mm/s]	Γ [mm/s]	η	χ
T 1 di-iodo-tyrosine pH 2	$1\,350 \pm 10$	0.30 ± 0.03	0.91 ± 0.02	0.05 ± 0.01	0.95 ± 0.04
T 2 di-iodo-tyrosine pH 10	$1\,268 \pm 10$	0.16 ± 0.03	0.96 ± 0.02	0.07 ± 0.01	0.93 ± 0.04
T 3 di-iodo-tyrosine crystallized	$1\,339 \pm 10$	0.30 ± 0.03	0.96 ± 0.02	0.05 ± 0.02	0.91 ± 0.04
DIT [8]	$1\,367 \pm 15$	0.30 ± 0.03	0.82 ± 0.08	0.07 ± 0.03	0.93 ± 0.07
V 1 TMV pH 3 tyrosine 139 iodinated	$1\,342 \pm 10$	0.20 ± 0.03	0.92 ± 0.02	0.07 ± 0.02	0.98 ± 0.04
V 2 TMV pH 8 tyrosine 139 iodinated	$1\,323 \pm 10$	0.17 ± 0.03	0.98 ± 0.02	0.06 ± 0.01	0.94 ± 0.04
V 3 TMV pH 8 tyrosine 139	$1\,344 \pm 13$	0.17 ± 0.03	0.96 ± 0.04	0.03 ± 0.03	0.98 ± 0.05
Cysteine 27	$1\,488 \pm 15$	0.54 ± 0.06	0.95 ± 0.06	0.04 ± 0.04	1.04 ± 0.08
RSI [11]	$1\,510 \pm 15$	0.41 ± 0.02	0.82 ± 0.04	0.07 ± 0.03	—

This is readily understood, as the molecule in the crystallized sample is obviously protonated. This result is supported by the measurements of Groves *et al.* [8] who also investigated crystallized tyrosine.

In the case of TMV it has to be assumed that a hydrogen bond at tyrosine 139 is at least partly responsible for the helical quaternary structure of the virus [9]. Since a hydrogen bond alters the *pK*-value of a group the determination of this value for tyrosine 139 in TMV is appropriate. In this case chemical methods are not suitable, because of the many ionisable groups in the protein. The Mössbauer method, however, is highly selective, since the sites of the resonator atoms are uniquely defined.

A comparison of the Mössbauer parameters obtained from the spectra for the free tyrosine molecule (T 1 : pH 2 ; T 2 : pH 10) and for tyrosine 139 in TMV (V 1 : pH 3 ; V 2 : pH 8) leads to the following conclusions :

1) Tyrosine 139 in TMV at pH 8 (V 2) is *not* non-protonated ; the *pK*-value for the hydroxyl group of iodo-tyrosine 139 in TMV is raised compared to that of free iodo-tyrosine.

2) The results for tyrosine 139 in TMV at pH 3 (V 1) do not confirm the assumed protonated state of the free molecule.

On being built into the TMV particle the OH-group of tyrosine 139 is obviously subject to a change, so

that its state cannot be described simply as protonated or non-protonated.

The formation of a hydrogen bond involving the hydroxyl group of tyrosine 139 in TMV can explain these findings. Additional investigations at higher pH-values are being performed in order to provide support for this conclusion. In particular, we wish to elucidate the relationship between rupture of the hydrogen bond and the destruction of the helical structure of the virus at higher pH-values.

With the measurement using absorber V 3 (tyrosine and cysteine iodinated) the first Mössbauer spectrum of a stable sulfenyl-iodine bond in cysteine has been recorded, and provides proof for the assumption that this otherwise unstable bond in cysteine is stable in TMV [10]. The parameters of the cysteine spectrum agree with those obtained by Potasek [11] who investigated a sulfenyl-iodine bond in the compound RSI.

In addition to the above mentioned results the asymmetry parameter η can also be obtained from the data. Its numerical value is close to zero for all samples. In other words : The electric field gradient shows only small deviations from rotation symmetry.

The parameter χ , which denotes the recoilless absorption anisotropy, is approximately 1, i. e. only small anisotropies are observed for the Debye-Waller-factor.

Additional measurements are being carried out, in order to determine the *pK*-value of iodo-tyrosine 139 in TMV.

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