

PROTON MAGNETIC RESONANCE STUDIES OF RIBONUCLEASE T₁

Assignment of histidine-27 C2-H and C5-H proton resonances by a photooxidation reaction

R. FÜLLING and H. RÜTERJANS

Institut für Physikalische Chemie der Universität Münster, 4400 Münster, FRG

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1. Introduction

Three histidine residues occur at positions 27, 40 and 92 in the amino acid sequence of RNase T₁ (EC 2.7.7.26) [1].

A photooxidation of RNase T₁ without inhibitor results in a quick destruction of more than one histidine and a loss of activity. This indicates that histidines are involved in the catalytic process [2]. In order to obtain some information about the functional properties of the histidines, NMR investigations were made [3–5]. One of the C2H proton resonances was assigned to His 40 using a combination of tritium and deuterium exchange techniques [5]. It could also be shown that His 40 is a part of the active site. However, these results obtained by exchange techniques did not appear to be quite satisfactory to prove the assignment of the remaining C2H and C5H imidazole proton resonances. In order to get the necessary information about His 27 and His 92 we modified RNase T₁ by photooxidation following [6]. Histidines of RNase T₁ were photooxidized in the presence of inhibitors [6]. However, the activity was found not to be affected. We were able to isolate the modified products by various chromatographic methods and identified one of the derivatives with His 27 photooxidized by both, NMR and peptide analysis. A newly developed isolation and purification procedure for RNase T₁ will be described also.

2. Experimental procedure

2.1. Materials

2'-GMP, Triton X-100, methylene blue, TPCK-

trypsin, fluorescamine and Servalyte were purchased from Serva, Heidelberg; GpA is a product of Waldhof, Mannheim; acrylamide, *NN'*-methylene bis-acrylamine and riboflavin were purchased from Biorad Laboratories, Richmond, CA. All other reagents were obtained from Merck AG, Darmstadt and were of reagent grade purity.

2.2. Determination of enzymatic activity

Guanylyl-(3'-5')adenosine, 1 mg, was dissolved in 15 ml 0.1 M Tris-HCl (pH 7.5) containing 0.002 M EDTA. To 3 ml of the substrate solution (in a 1 cm optical cell) 10 μ l enzyme was added. The reaction was followed by ΔA_{280} in a recording spectrophotometer (Gilford spectrophotometer 250) at 25°C [7].

2.3. Purification of RNase T₁

1. First concentration step

RNase T₁ was isolated from 200 liter water extract of *Aspergillus oryzae*. After further dilution with 200 liter water, 1 kg DEAE-Sephadex A25 (Pharmacia, Uppsala) was added, the solution was adjusted to pH 7.5 with NaOH and then stirred for 1 h. The ion-exchange resin was separated from the solution by filtration and washed with 20 liter 0.01 M Na₃-citrate-HCl buffer (pH 5). The enzyme was eluted from the resin at pH 2.7 with 0.01 M Na₃-citrate-HCl buffer.

2. Second concentration step

The RNase T₁ containing extract was given to a Sephadex SP-C25 column (5 × 10 cm) which was equilibrated with 0.01 M Na₃-citrate-HCl buffer (pH 2.7) and eluted with 0.01 M Na₃-citrate-HCl (pH 7.5). The T₁-containing fraction (some 100 ml)

was brought to pH 3.8 with HCl. By this treatment the larger part of a brown dye of *Aspergillus oryzae*, which was accumulated in this fraction, precipitated and was removed by centrifugation. In the next step the solution was adjusted to pH 3.2 and centrifuged a second time.

3. Chromatography with a pH gradient elution

The solution was applied to Sephadex SP-C25 column (2.6 × 15 cm), equilibrated with 0.01 M Na₃-citrate-HCl buffer (pH 3.2) and the material was eluted with a pH gradient solution. The mixing chamber was filled with 600 ml 0.01 M Na₃-citrate-HCl buffer (pH 3.2) and the second chamber with 2000 ml 0.01 M citrate-HCl buffer (pH 4.2). Each of the steps II and III removed a lot of the brown dye present in *Aspergillus oryzae*, but the T₁-containing fraction was still dark-brown coloured. Further ion-exchange chromatography steps did not remove the dye completely. Therefore a sort of affinity chromatography was developed to almost completely remove the dye.

4. The RNase T₁-containing solution was adjusted to pH 3.2 with HCl and given to a SP-C25 column (1 × 20 cm), equilibrated with 0.01 M Na₃-citrate-HCl (pH 3.2). In the next step the column was washed with the same buffer, to which 1 mg/ml 2'-GMP was added. 2'-GMP is a strong inhibitor of RNase T₁. The complex of RNase T₁ with 2'-GMP has a different charge number as compared with RNase T₁. On Sephadex-SP it is only weakly bound at pH 3.2 and emerges very quickly in the effluent. The brown dye and other proteins are not affected by 2'-GMP and are therefore not eluted. The effluent is colourless, and the RNase T₁ is free of any protein contamination.

5. The 2'-GMP-containing RNase T₁ solution was adjusted to pH 2.5 with HCl and given to a small SP-C25 column (1 × 10 cm), equilibrated with 0.01 M Na₃-citrate-HCl buffer (pH 2.5), washed with 50 ml 0.01 M NH₄-acetate, adjusted to pH 2.5 with HCl and eluted with 0.01 M NH₄-acetate. The RNase T₁ emerged in a small volume free of nucleotide and inorganic phosphate.

6. The protein was desalted by gel-chromatography on a Sephadex G25 column (2.6 × 30 cm), equilibrated with doubly distilled water, and lyophilized. During the steps 5 and 6 all glassware was carefully rinsed with conc. HCl to remove trace amounts of

inorganic phosphate [8]. RNase T₁ very strongly binds inorganic and organic phosphates and even if there is only a small amount of phosphate on the glass surface, the enzyme is accumulating phosphate during the isolation steps. Gel chromatography was not sufficient to remove nucleotides and inorganic phosphates as could be tested by a sensitive phosphate determination (malachite green method [8]). The purity of the proteins was checked by isoelectrofocusing on a Multiphor 2117 (LKB, Bromma, Sweden) in acrylamide gel (pH range 2.5–6; ~0.1 pH/cm) [9].

2.4. Amino acid analysis

Peptide samples (1–2 nmol) were hydrolyzed in 100 µl 6 N HCl for 24 h. The analysis was performed with an automated Beckman (121 M) amino acid analyser by a one-column procedure.

2.5. End group determination

N-terminal amino acids of peptide aliquots (0.5–5 nmol) were identified as dansyl derivatives on polyamide sheets (Schleicher and Schüll, Dassel) [10].

2.6. NMR measurements

For the NMR measurements the protein was dissolved in D₂O (pH 6.0) (99.75% D₂O) and heated to 50°C for 1 h and then lyophilized to remove exchangeable NH-protons. The pH was adjusted with DCl or NaOD. Reported pH values were direct meter readings (pH-Meter 26, Radiometer, Copenhagen). NMR spectra were obtained at 270 MHz with a Bruker WH 270 in the Fourier Transform mode. Chemical shifts are given in ppm from the internal standard TSP (2,2,3,3-tetradeutero-trimethylsilyl propanoic acid).

2.7. Photooxidation of RNase T₁ in the presence of inhibitor

A mixture consisting of 2 ml 1% methylene blue, 50 ml 0.1 M Tris-HCl (pH 7.5) containing 50 mg RNase T₁ and 75 mg 2'-GMP was illuminated from 10 cm distance by a 150 W spot light [6] for 3 h. The photooxidized enzyme solution was passed through an Amberlite IRC-50 (Serva, Heidelberg) column (1 × 1 cm), equilibrated and washed with 0.1 M Tris-HCl (pH 7.5) to remove the methylene blue. The Tris-HCl buffer and the bulk of the nucleotide

was removed by gel chromatography on Sephadex G-25 equilibrated and eluted with 0.01 M $\text{Na}_3\text{-citrate}$. The photooxidized derivatives were fractionated by gradient chromatography (as described for the native enzyme, step III) into three peaks. The greatest peak B was further treated in the same manner as described in step 5 to separate an active fraction (B2) from an inactive one (B1). The active fraction (B2) was concentrated, desalted and lyophilized in the same manner as described in steps 5 and 6 (purification of the native enzyme).

2.8. Peptide analysis of the modified protein

Photooxidized RNase T_1 (derivative B2), 2 mg, was dissolved in 250 μl 2 M urea; 0.1 M Tris-HCl (pH 8.5) and TPCK-trypsin was added. The cleavage was performed for 12 h at 37°C. The peptides were isolated by gel chromatography on Sephadex G-50, equilibrated and eluted with 0.1 M NH_4HCO_3 . Peptide Trp.II (26–41) (50 nmol) was further digested with thermolysin (Merck, Darmstadt) (1% in 0.1 M NH_4HCO_3 , 2 mM CaCl_2) for 3 h at 45°C. The peptide mapping of the thermolytic peptides was performed on cellulose TLC-plates (20 \times 20 cm), Schleicher and Schüll, Dassel: first dimension, electrophoresis at pH 6.5; second dimension, ascending chromatography in pyridine/butanol/water (35/35/30, v/v/v) [11].

3. Results and discussion

The native enzyme which has been isolated in good yield (80%) was identified as being pure RNase T_1 by amino acid analysis, ultraviolet measurement and by the determination of the enzymatic activity [12]. Furthermore no differences to the commercially available RNase T_1 (Sankyo Co., Tokyo) has been detected. In the reaction mixture native enzyme and a small amount of inactive derivatives with more than one histidine photooxidized was found beside the main fractions B1 and B2 (each of which have lost one of the three histidines). The B1 fraction has lost also its activity towards the splitting of GpA. It has not yet been the subject of further investigations. The fraction B2, on the contrary, has kept full enzymatic activity. The analysis of derivative B2 revealed that His 27 in the sequence is photooxidized.

The only tryptophan residue is obviously not affected by photooxidation as could be shown by the almost identical ultraviolet spectra of both derivatives B1 and B2 with respect to those of the native enzyme.

Comparison of the $^1\text{H-NMR}$ spectra of the derivative B2 with the $^1\text{H-NMR}$ spectra of native RNase T_1 revealed that one of the imidazole C2-proton resonances is lost during photooxidation (fig.1,2). This signal must be assigned to His 27. The remaining C2-PMR signal in the absorption range of 8–9 ppm has been assigned to His 40 [5]. This histidine is supposed to be located in the active site of RNase T_1 , since its imidazole C2 proton resonance is found to shift position on the addition of nucleotides [4]. The anomalous pH dependence of the chemical shift of both C2 proton resonances has been interpreted as being due to interactions of the two imidazole rings with carboxylate groups of the enzyme. With photooxidation one of the C5 proton resonances has also been lost which should therefore be assigned to His 27 (fig.1,2). It is open to question whether a second resonance in that absorption region (7.2–7.7 ppm) can be assigned to the C5 proton resonance of His 40 or whether this resonance has to be assigned to the

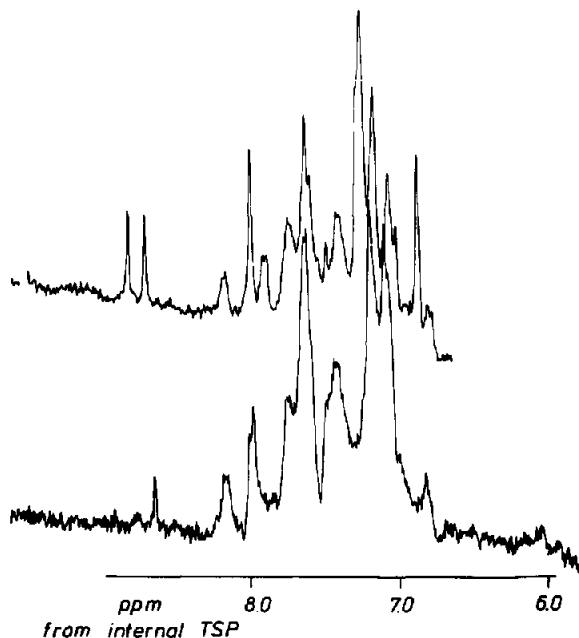


Fig.1. Absorption region of the ring proton resonances of aromatic amino acids of RNase T_1 (upper spectrum) and of photooxidized RNase T_1 (lower spectrum) at pH 5.15.

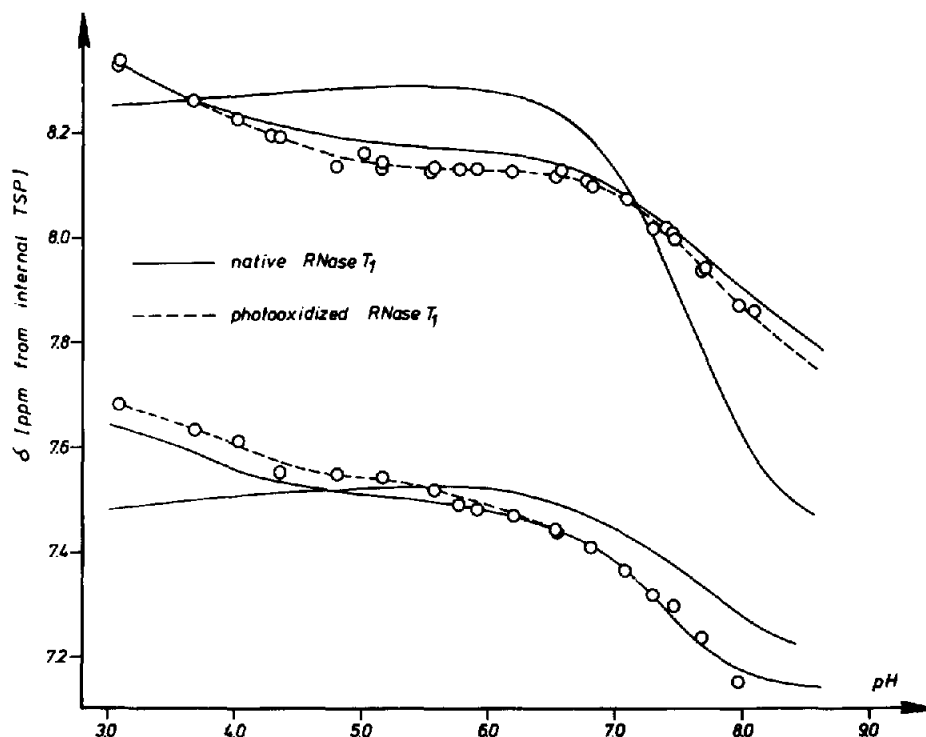


Fig.2. pH dependence of the chemical shifts of the histidine C-2 and C-5 proton resonances of native RNase T₁ and of photooxidized RNase T₁.

C2 proton of His 92. Further studies are necessary to settle that question. Also changes in the absorption region of the other aromatic amino acids which occur upon photooxidation of His 27 still have to be interpreted.

To conclude, it could be shown that photooxidation of histidines with a subsequent peptide analysis of the derivatives can be useful in the assignment of imidazole C2 and C5 proton resonances of proteins.

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References

- [1] Takahashi, K. (1971) *J. Biochem. (Tokyo)* 70, 945–960.
- [2] Irie, M. (1970) *J. Biochem. (Tokyo)* 68, 69–79.
- [3] Rüterjans, H. and Pongs, O. (1971) *Eur. J. Biochem.* 18, 313–318; Rüterjans, H., Witzel, H. and Pongs, O. (1969) *Biochem. Biophys. Res. Commun.* 37, 247–253.
- [4] Rüterjans, H., Maurer, W., Fülling, R. and Both, V. (1976) in: *Ribosomes and RNA Metabolism*, vol. 2, pp. 109–118, Slovak Acad. Sci., Bratislava.
- [5] Arata, Y., Kimura, S., Matsuo, H. and Narita, K. (1976) *Biochem. Biophys. Res. Commun.* 73, 133–140.
- [6] Waku, K. and Nakazawa, Y. (1970) *J. Biochem.* 68, 63–67.
- [7] Zabinski, M. and Walz, F. (1976) *Arch. Biochem. Biophys.* 175, 558–564.
- [8] Hess, H. and Derr, J. (1975) *Anal. Biochem.* 63, 607–613.
- [9] Karlson, C., Davies, H., Öhman, J. and Anderson, U. B. (1973) Application note, LKB-Producter AB, Stockholm, Sweden.
- [10] Hartley, S. (1970) *Biochem. J.* 119, 805–822.
- [11] Beyreuther, K., Raufuss, H., Schrecker, O. and Hengstenberg, W. (1977) *Eur. J. Biochem.* 75, 275–286.
- [12] Egami, F. and Nakamura, K. (1969) in: *Microbiol. Ribonucleases*, p. 22, Springer, Berlin, Heidelberg, NY.