Investigation of the Hardening Reaction of Gelatin with ^{13}C Labeled Formaldehyde by Solution and Solid State ^{13}C NMR Spectroscopy

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Model reactions of the hardening of gelatin with ^{13}C labeled formaldehyde were monitored by ^{13}C NMR spectroscopy in solution and in the solid state. In solution primary attack of formaldehyde first forms methylols of lysine residues and later of arginine residues, which react to a lysine-arginine crosslink. During the drying process of hardened gelatin, in addition to the lysine-arginine crosslink an arginine-arginine crosslink is formed. The rate of crosslink formation is strongly influenced by humidity.

Introduction

Gelatin is one of the last natural products which is used since the last century in the preparation of photographic films. Despite its widespread use as a photographic gelatin the mechanism of the hardening reaction with formalin is not fully understood [1–4]. This reaction is used to reduce swelling and to increase the melting point of the photographic material.

The composition of formalin solutions has been intensively studied by NMR spectroscopy [5, 6]. These investigations gave unambiguous assignments of the ^{13}C NMR chemical shifts of formaldehyde oligomers. NMR studies of model reactions of formaldehyde and various amino acids gave the ^{13}C chemical shifts of reaction products such as methylols and crosslinks [7]. The NMR investigation of the crosslinking reaction of gelatin and formaldehyde [6, 7] revealed that in solution, primary attack of formaldehyde occurs at amino acid residues lysine and arginine to form methylols and later the corresponding methylene bridge. We were interested in the kinetics of the hardening reaction under various experimental conditions. Initial experiments in solution could be followed by high resolution ^{13}C NMR spectroscopy whereas data of dried products required the use of ^{13}C CP/MAS NMR spectroscopy.

Experimental

Chemical and material

To yield solutions of 6% gelatin in H_2O , 600 mg gelatin (Rousselot, France) were allowed to swell 10 min in 5 ml distilled water, then dissolved at 38°C with further 4.4 ml H_2O for solid state experiments of 3.4 ml H_2O and 1 ml D_2O for solution experiments. Here, 4 ml of this solution were transferred to a 10 mm NMR tube and degassed in an ultrasonic bath. Hardening was induced by addition of $320\ \mu\text{l}$ 0.5% $^{13}\text{CH}_2\text{O}$ in H_2O (Merck, Sharp & Dohme, Canada). Samples for solid state experiments were prepared in a box under defined conditions of air humidity and temperature. To the gelatin slurry in a Petri disk, $100\ \mu\text{l}$ of 5% $^{13}\text{CH}_2\text{O}$ (Merck, Sharp & Dohme, Canada) were added. After a certain reaction time the hardened gelatin was transferred to a double-air bearing rotor made of Al_2O_3 (7 mm O. D.).

NMR measurements

^{13}C NMR spectra (50 MHz) were carried out using a Bruker MSL 200 NMR spectrometer. Solution spectra were measured in 10 mm NMR tubes by accumulating up to 40 K transients at a pulse repetition time of 1 s. Reference was the signal of external 3-(trimethylsilyl)-3,3,2,2-tetradeuteropropionic acid Na salt. Solid state NMR spectra were obtained with samples of 200–300 mg in double-air bearing rotors of Al_2O_3 by accumulating up to 1 K transients at spinning rate of 3000–3500 Hz. Typically, a proton 90° pulse length of $6\ \mu\text{s}$ together with a contact time of 3 ms and a pulse repetition time of 4 s was employed. The spectra were externally referenced to liquid tetramethylsilane.

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Results and Discussion

A. Solution state ^{13}C NMR investigations

Fig. 1 shows the ^{13}C NMR spectra of a 12% gelatin solution hardened with 0.5% ^{13}C labeled formaldehyde in time courses directly after addition of the ^{13}C formaldehyde (time course 0–1 h, Fig. 1a) and after 71 h of hardening reaction (time

Fig. 1. Solution state ^{13}C NMR spectra of 12% gelatin hardened with 0.5% ^{13}C formaldehyde recorded in two time courses. a) 0–1 h, b) 71–72 h.

course 71–72 h, Fig. 1b). Fig. 1a is governed by the resonances at 88.6 ppm, 84.9 ppm and 74.4 ppm, which can be assigned to formalin dimer **B**, to formalinhydrate **A**, and to the lysinemethylol **N**, respectively [5–7]. In addition to these signals in Fig. 1b, further main resonances at 70.7 ppm and 62.3 ppm appear, which are due to argininemethylol **O** and the arginine-lysine crosslink **P** [6, 7]. This finding is consistent with previous investigations revealing that the main attack of formaldehyde takes place at the amino residues of arginine and lysine [6, 7]. Even both amino acids are present to minor extent in gelatin the main reaction of formaldehyde takes place at their terminal amino groups for two reasons: In the case of lysine, the steric accessibility as well as the mobility of the terminal amino group favours the reaction with formaldehyde. In the case of arginine, the formation of an intramolecular hydrogen bond also accelerates the reaction of the guanidino group (Fig. 2). In a second step of the crosslinking reaction a lysine-arginine crosslink **P** is formed, which is stabilized by an intramolecular hydrogen bond (Fig. 2). It is interesting to note that lysinemethylol **N** is formed immediately after addition of formalin whereas the formation of argininemethylol **O** and the lysine-arginine crosslink **P** is delayed. In detail, Fig. 3a–c show the signal intensity behavior of **O**,

Fig. 2. Structure of the main methylols and crosslinks formed in the hardening reaction of gelatin and formaldehyde.

a) LYSINEMETHYLOL **N** c) LYSINE / ARGININE CROSSLINK **P** b) ARGININE-METHYLOL **O**

Fig. 3. Time course of the main ^{13}C signals of reaction products formed after addition of 0.5% ^{13}C formalin to a solution of 12% gelatin. a) Lysinemethylol **N**, b) argininemethylol **O**, c) lysine-arginine crosslink **P**.

N and **P** during the hardening reaction of 12% gelation with 0.5% ^{13}C labeled formalin over a time period of 71 h with a time resolution of 43 min. Under the experimental conditions in a 10 mm NMR tube a large excess of formalin is present because of limited surface area and the impossibility of applying a rigorous drying procedure. During the time course of the hardening reaction the amount of lysinemethylol **N** is decreasing continuously whereas simultaneous formation of arginine-methylol **O** together with the reaction to lysine-arginine crosslink **P** is taking place. Because of the excess of formaldehyde a maximum of the arginine-methylol resonance in the reaction course is not

detected. Thus the rate constant of the arginine-methylol formation governs the whole hardening reaction in solution.

B. Solid state ^{13}C NMR investigations

Fig. 4 shows a solution and a solid state NMR spectrum of gelatin hardened with ^{13}C formalin. It is clearly seen that all signals of the main species formaldehyd-hydrate **A**, formaldehydedihydrate **B**, argininemethylol **O**, lysine-arginine crosslink **P** involved in the hardening reaction absorb at the same chemical shift values in the solution (Fig. 4a) and the solid state (Fig. 4b) NMR spectra. There-

Fig. 4. ^{13}C NMR spectra of hardened gelatin. a) Recorded in a 10 mm NMR tube, b) recorded in a double-air bearing rotor.

fore all assignments performed in the high resolution spectra can be adopted in the solid state investigations. Because of the difficulties of removing all dipole-dipole interactions in the solid state NMR spectra of gelatin, the spectral resolution is lower compared to the solution spectra. This may be one reason why the signal of lysinemethylol **N** at 74 ppm is not clearly detected besides the broad signal of argininemethylol **O** at 70 ppm. The second reason is the relative intensity of the lysine and the argininemethylol signals. Because the amount of lysinemethylol is decreasing in the hardening reaction whereas the amount of argininemethylol is increasing as far as formaldehyde is present, the signal of argininemethylol **O** must be more intense than the signal of lysinemethylol **N**. Therefore in Fig. 4b the resonance of **N** is expected to adsorb under the left shoulder of the signal of **O**. Whereas the solution state spectra of gelatin hardening are governed by the signals of **N**, **O** and **P**, in the solid state spectra an additional signal **T** at 60 ppm is detected. This observation together with the absence of an intense signal of **N** indicates that there

are different reaction conditions in a 10 mm NMR tube and a rotor used in solid state experiments. Whereas in all solution state experiments an excess of formaldehyde is present, in the solid state experiments the final reaction state can be observed. Fig. 5 shows the spectrum of 6% gelatin hardened with 5% ^{13}C formalin in two time courses of 2–3 h and 18–19 h at 303 K (60% air humidity). Comparing Fig. 5a and 5b and taking the gelatin signal at 43 ppm (Gly C_α) as internal standard the progress in the hardening reaction is clearly seen. The signals of the formaldehyde monohydrate **A** and dihydrate **B** disappeared whereas the signals of the units **P** and **T** increased twice. The argininemethylol resonance **O** remained constant in intensity. This finding clearly proves that the resonances of **P** and **T** are caused by crosslinks and that the arginine residue is playing an important part in the crosslinking reaction. Therefore the signal at 60 ppm is assigned to an arginine-arginine crosslink **T**. In solution the chemical shift value of this crosslink is 56 ppm [7]. Due to the formation of intramolecular hydrogen bonds in the dried gelatin

Fig. 5. Solid state ^{13}C NMR spectra of 12% gelatin hardened with 0.5% ^{13}C formaldehyde at an air humidity of 60%. Spectra were recorded in two time courses of a) 2–3 h, b) 18–19 h.

the observed downfield shift between solution and solid state spectra may be explained.

In order to simulate differing production conditions gelatin hardening experiments with different amounts of air humidity were conducted. Experiments were carried out with a special preparation box at air humidities of 40% and 100%. At air humidity of 40% a solid state spectrum of the resulting gel was recorded immediately after the addition of formalin. The formaldehyde was present in the gelatin as dihydrate **B** and only small amounts of **O** and **P** resonances could be detected. Even after 24 h of hardening reaction without any heating the amounts of argininemethylol **O** and crosslink **P** did not increase severely. When an air humidity of 100% was employed, the NMR signals of all species showed very small halfwidths owing to increased mobility in the resulting gelatin gum. Under these reaction conditions mainly arginine-arginine crosslinks **T** are formed after 24 h of hardening at 303 K (Fig. 6a). An additional signal at 65 ppm is due to terminal CH_2OH -groups. After 74 h of drying in the preparation box, the signals of **A** and **N** decreased whereas the signals of **T** and the terminal CH_2OH increased. Final drying at 333 K over three days led to a species composition shown in Fig. 6b. Three signals of **O**, **P** and **T** can be detected. This finding is consistent to that of Fig. 4b, which was obtained at an air humidity of 60%. At a higher air humidity in a first crosslinking reaction **T** crosslinks are formed. In a second step **P** crosslinks are also built leading to a final crosslink composition independent of the preparation procedure. However, different crosslinking species levels are maintained during the hardening reaction. Thus it is clear that a partially hardened film has another behaviour than a complete dry film. Low humidity favours the oligomerization of formaldehyde versus the crosslinking reaction. On the other hand, high humidity strongly retards the hardening reaction, leading to high amounts of unreacted formaldehyde in the gelatin. Optimum

crosslinking is achieved at moderate humidities, *e.g.* 60–70% air humidity.

Fig. 6. Solid state ^{13}C NMR spectra of 6% gelatin hardened with 2.5% ^{13}C formaldehyde at an air humidity of 100%. Spectra were recorded at time courses of a) 24–26 h, b) 144–144.5 h.

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