Temporary Changes in the dc Electrical Conductivity of MX (3-Chloro-4(Dichloromethyl)-5-Hydroxy-2(5H)- Furanone) Treated Collagen

LESZEK KUBISZ^{1*} and PRZEMYSŁAW ANDRZEJEWSKI²

¹*Department of Biophysics, Karol Marcinkowski University of Medical Sciences in Poznań, Poland*

²*Department of Water Treatment Technology, Adam Mickiewicz University, Poznań, Poland*

(*Author for correspondence, e-mail: kubisz@main.amu.edu.pl)

Abstract. The influence of MX (3-Chloro-4(Dichloromethyl)-5-Hydroxy-2(5H)- Furanone), a strongly mutagenic compound, on the temperature dependence of the dc electrical conductivity of collagen as a function of time was studied. Collagen was immersed in MX solution, next dried and pressed into tablets. The MX concentration was measured by HPLC analysis. The reduction of MX concentration to 10% of the initial value was observed in the presence of collagen in the solution, whereas in the control solution concentration of MX decreased to 70% of the initial value. Measurements of electrical conductivity were performed for the temperature range 295–453K and activation energies for the charge conduction process were calculated. Within the temperature range 295–340K, the presence of MX decreased electrical conductivity of collagen. Calculated activation energies were typical for dry proteins. Within the temperature range 295–320K activation energy decreased with time, probably due to the stronger interactions in the collagen-water-MX system. For temperatures between 320–410 and 430–450K the activation energy was not time dependent and the application of MX did not change the structure of the collagen macromolecule. The temporary changes occurring at the lower temperatures being due solely to changes in the collagen-water interactions.

Key words: 3-Chloro-4(Dichloromethyl)-5-Hydroxy-2(5H)-Furanone, activation energy, collagen, electrical conductivity, MX

1. Introduction

Collagen is a component of almost all tissues. The electrical properties of collagen in its solid state depend on the water content [1, 2]. Thus, the electrical conductivity, dielectric losses and activation energy of the charge conduction process of collagen all depend on the water content [1, 3, 4]. Modifications in the collagen – water system result in changes in electrical conductivity and thermal stability. Changes in thermal stability can be shown by either an increase or a decrease in the temperature at which denaturation occurs [3, 5–7].

Collagen can be modified by γ -irradiation, heating, high pressure or the effect of different chemical compounds. Another method is that based on the possibility of attaching naturally occurring chemical compounds, as with hydroxyapatite in the case of bone. An analogous method can be utilized for the fabrication of a biomaterial similar to collagen but with new physicochemical properties [8]. A particular interest is in a compound that influences the properties of collagen even in weak concentrations. The strong mutagen 3-Chloro-4(Dichloromethyl)-5-Hydroxy-2(5H)-Furanone (known as MX) belongs to this group [9]. It has been shown that MX decreases the dc electrical conductivity of collagen in the solid state and makes more difficult water liberation [10]. These results can be explained by the electrophilic properties of MX [11, 12]. Probably either takes place the reduction of the number of specific sites at which water molecules can be bound to collagen, or stronger interactions in collagen-water-MX system are created. On the other hand, for solid-state proteins, electrical conductivity depends on the water content [2] and according to Nomura et al. [13] and Bigi et al. [14] four different regimes of hydration of collagen are characterised by different activation energy of water removal. Thus expected changes in collagen-water system due to the presence of MX should be confirmed by an increase in activation energy of the charge conduction process and a decrease of electrical conductivity of collagen.

The physical properties of MX depend on temperature and pH [15, 16]. The stability of MX in water at pH 8 is relatively high, but finite, with a half-life time of 6 days at 293K [17]. 100% of MX removal was observed during 1-hour heating in boiling water at the temperature of 373K [16]. The stability of MX molecules suggests a time-dependent influence on the electrical properties of collagen. In the present study the relationship between activation energy and time was studied.

2. Material and method

The material used was bovine Achilles tendon (BAT) insoluble collagen, type I, purchased from the Sigma Chemical Company. MX was synthesised by the method developed by Padmapriya et al. [18] and stored at 277K with ethyl acetate [19]. A dose of MX, equal to $7.5 \mu\text{g}$ per 100 ml of distilled water, was administered to 40mg of bovine Achilles tendon collagen. For comparison, the control solution of MX without immersed collagen was prepared dissolving $7.5 \mu\text{g}$ of MX in 100 ml of distilled water. The dose chosen was smaller than the orally toxic dose and larger than those causing changes in blood plasma [20, 21]. Control samples were prepared from collagen immersed in distilled water. The pH of the solution, in which the samples were immersed, was measured two times, first, after the immersion of the material and second time after 2 days. For controls changed from 7.4 to 7.7 and for MX from 7.7 to 8.0.

All the specimens were immersed for two days at a temperature of 277K. Next, the collagen was dried for two days at a temperature of 295K and pressed into tablets using the tableting machine with controlled pressure (1MPa). Samples were

stored from 2 to 14 days at a temperature of 277K. For 24 hours before starting the experiment each sample was covered with silver paste electrodes and kept at 295K in a measuring device.

Two days after the immersion of collagen into solution, the concentration of MX was measured according to own methodology based on HPLC Waters equipment. Because analyzed matrix was simple, MX and collagen only, analytical procedure without derivatization and cleaning procedure can be used. Each sample of water were acidified to pH=2 with hydrochloric acid prior to HPLC analysis. The apparatus used was HPLC WATERS 2690 coupled with Waters 486 UV-VIS detector (200–600 nm) and Waters C18 column (300 mm × 3 mm i.d.). Parameters of HPLC were as follows: injection volume 10 μ l, eluent: gradient mode, methanol – water 10:90 to 60:40, flow 0.7 ml/min, detection: UV – 271 nm, data processing system: CSW ver.1.7.

The water content in collagen was measured by weighing at the temperature 295K and after 1-hour heating at the temperature of 383K under atmospheric pressure.

Applying a constant potential difference to each specimen and measuring the resultant current flow obtained the dc electrical conductivity for the collagen samples. The method applied in this experiment was described in detail in previous articles [10, 22].

Measurements were performed under normal pressure, in the temperature range 295–453K during linear temperature changes. Samples were exposed to two heating runs. The first run, starting from ambient temperature was finished at temperature 383K. The samples were kept at this temperature for about 1 hour, to remove free water, and then allowed to cool to the temperature of 295K. Simultaneously, heating at the temperature of 373K gave the thermal decomposition of MX [16]. In the second run, the samples were heated from the ambient temperature to 453K.

3. Results and discussion

The measurements of the concentration of MX based on HPLC gave as follows: in the control solution MX concentration decreased to 70% of initial value. In the case of the solution where collagen was immersed, the concentration of MX decreased to 10% of the initial value. The difference in the final concentration was probably due to the adduction of the MX molecule to the collagen structure. However a transformation of MX molecule under the influence of collagen is not excluded.

No significant difference in water content for MX samples and control samples was observed. Water content of MX treated collagen was $11.0 \pm 0.8\%$, whereas for controls the mass loss was $10.8 \pm 0.4\%$. This result confirmed, expected earlier, stronger interaction in collagen-water-MX system than the reduction of the number of specific sites of the collagen macromolecule where water can be bound.

Figures 1 and 2 show the typical temperature dependence of the dc electrical conductivity (σ) of the MX treated collagen and the control samples, respectively.

Figure 1. Temperature dependence of dc electrical conductivity of MX treated collagen.
 Legend: + = MX treated collagen, first heating; □ = MX treated collagen, second heating.

Figure 2. Temperature dependence of dc electrical conductivity of control samples.
 Legend: × = Control samples, first heating; ○ = Control samples, second heating.

Figure 3. Arrhenius plot for electrical conductivity.

Legend: + = MX treated collagen, first heating; □ = MX treated collagen, second heating; x = Control samples, first heating; ○ = Control samples, second heating.

All samples were submitted to two consecutive heating runs. During the first heating run (295–383K), for MX-treated samples the peak of electrical conductivity σ was observed at the temperature of 315K and for controls at 319K. In the temperature range 295–383K, the presence of MX reduced the electrical conductivity of collagen samples with respect to the controls. Moreover the peak of dc electrical conductivity was shifted from 315K to 319K. According to Tomaselli the peak at 315K and 319K can be explained as the superposition of the transition of water molecules from bound to free state and the diffusion of water molecules out of the collagen-water system [23]. The shift was the result of collagen-water-MX interaction and indicated indirectly more difficult water liberation from the system.

During the second run characteristic peaks at 315 and 319 K disappeared and a uniform increase in σ with temperature was observed. Up to 330K the electrical conductivity of the MX treated collagen was significantly lower than that of the controls.

On the basis of these results the activation energy E for a charge conduction process could be determined according to the following well-known formula for the thermally activated processes [24]:

$$\sigma(T) = \sigma_0 \exp\left(-\frac{E}{kT}\right)$$

where σ stands for dc electrical conductivity, T for temperature and k for Boltzmann's constant. The calculations were performed at the intervals of linear dependence of

Table I. Activation energy, first heating run

Temperature range (K)	Activation energy (kJ/mole)			
	Collagen + MX		Controls	
	x±s	range	x±s	range
290–320	55±10	40–64	40±2	22–67

Table II. Activation energy, second heating run

Temperature range (K)	Activation energy (kJ/mole)			
	Collagen + MX		Controls	
	x±s	range	x±s	range
290–320	≈90	–	56±2	54–58
350–410	46±5	41–51	50±6	41–57
430–450	124±8	108–126	114±7	106–120

$$\ln \sigma = f(1/T)$$

[24] with the correlation coefficient r no smaller than 0.99. Fig 3. shows the Arrhenius plots used to calculate the activation energies of both MX-treated collagen samples and the control samples. Calculations were made for the first heating at 290–320K, for the second heating at 290–320K, 350–410K and for temperatures of 430–450K. The averaged activation energies of the charge conduction process, their standard deviations and ranges, defined as limits within which activation energy varies, are summarized in Tables I and Table II. The values of activation energy were smaller than 260kJ/mole, indicating that electrical conductance is not connected with the charge carriers injected from the electrodes [25].

The non-linearity of relationship (4) (see Figure 3) probably indicates that, the conduction process exhibited a non-Arrhenius thermal activation. This can be explained by the multiple denaturation in solid-state collagen. According to Okamoto [26], solid-state, type 1 collagen consists of three regions of different structure: an amorphous region, with phase transition at 393K, less oriented, and having a widely distributed denaturation point between 353–373K and 453 K, and a stable crystalline region, having denaturation temperatures around 473K. However, a higher temperature (490K) of dry collagen denaturation point was reported [27]. Furthermore, σ -T curve reflected the presence of water and other organic components of bone. According to Nomura et al. [13] four different regimes of hydration in collagen have been identified: first (0.00–0.07 g water/g collagen) is associated

Figure 4. Activation energy vs. storage time, temperature range 290–320K.

Legend: + = MX treated collagen, first heating; □ = MX treated collagen, second heating; × = Control samples, first heating; ○ = Control samples, second heating.

with structural water; the second (0.07–0.25 g/g) with bound water; third (0.25–0.45) the transition regime in which both bound and free water are absorbed and fourth (>0.45 g/g) with free water. Bigi et al. [14] reported the complete removal of bound water from a sample heated up to 333 K and the weight loss of about 9%, and removal of structural water in two steps: first, progressive (about 14%) obtained by heating the sample up to 373K and second, when the remaining structural water is removed, at the temperature range of 373–443 K. According to Pineri et al. [28], activation energy of the third regime of hydration is about 40kJ/mole. For the bound water regime he distinguished two sub-ranges: 0.07–0.11 g/g with activation energy of 75 kJ/mole and 0.11–0.235 g/g with activation energy 54 kJ/mole.

In this experiment the mass loss of sample was about 10%, what indicated a presence of structural and bound water. For controls and MX samples their activation energy, in the temperature ranges of 290–320K for the first heating run, was close to the activation energy of bound water. Second heating resulted in increased activation energy, but for controls their energy was still in the range of activation energy of bound water. For MX samples, considering the results shown in Figure 3, estimated activation energy was equal about 90 kJ/mole. This can be explained by water loss due to heating up to 383 K, under atmospheric pressure reducing the amount of water. An increase in activation energy of solid-state proteins with the decreased water content is well known [2].

The wide range of activation energy levels and the relatively short half-life time for MX [17] suggest that activation energy may depend on the length of

sample storage time. Figure 4 shows the activation energy-time relationship for the collagen-MX samples and the control samples for both the first and second heating runs. With the first heating run (temperature range 290–320K) the activation energy in the collagen-MX system diminished with time and for the controls opposite tendency was observed. Such a relationship can be explained by the varying the amount of water present in collagen. Because no significant difference in water content of MX samples was observed, probably the presence of MX disrupted the process of transition of water molecules from bound to free state and made difficult for the evaporation of water out of collagen.

Within the temperature range of 350–410K, activation energy for both MX and control samples was in the range of liberation of third kind of water, probably the transition from the bound state to free state and evaporation took place. For the temperature range of 430–450K, activation energy was comparable with this reported by Pethig for dry proteins as 135 kJ/mole [2]. Moreover, in the temperature range 353–453K, the denaturation of less oriented crystalline region of collagen take place. Obtained, within these two temperature ranges, activation energy was time independent.

The difference in electrical conductivity and activation energy between MX and controls can be explained in terms of stronger interactions in collagen-water-MX system. Because MX is not a stable chemical compound [16, 17], and number of MX molecules declined with time, water can be liberated reducing simultaneously activation energy and increasing electrical conductivity.

However, the temperature range 430–450 K did not reveal a time-dependent level of activation energy for either the controls or the collagen-MX samples. Therefore MX does not influence the amount of structural water in collagen. MX influences the electrical properties of collagen at temperatures below 380K at which MX loses its mutagenic properties due to thermal decomposition [15,16]. All the activation energies observed are within the range typical for dry collagen [2].

4. Conclusions

The results reported here indicate that the strongly mutagenic compound MX influences the electrical conductivity of collagen. MX reduces electrical conductivity collagen-water system at temperature range of 290–320K. The fall in activation energy with time suggests that the influence of MX is a temporary effect. The absence of changes of electrical conductivity, at higher temperatures, shows that MX has no influence on the structure of the collagen molecule itself, the temporary changes occurring at the lower temperatures being due solely to changes in the collagen-water interactions. The dc electric conductivity method is useful in studies on electrical properties of dry proteins containing low-level impurities.

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