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Freezing of water bound in lichen thallus as observed by ^{1}H-NMR. I. Freezing of loosely bound water in *Cladonia mitis* at different hydration levels

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Abstract

Proton NMR spectra for *Cladonia mitis*, hydrated to $\Delta m/m_0 = 0.193$, 0.126 and to 0.076, were recorded at different temperatures between room temperature and −45 °C. The loosely bound and free water fractions were selected for the observation using very long pulse length ($\pi/2 = 35$ μs). For the thallus hydrated to $\Delta m/m_0 = 0.193$ the stepwise increase in linewidths and decrease of area under peak caused by free water freezing was observed at $t = -20$ °C, whereas for thallus hydrated to lower level the decrease in area under peak proceeded continuously with decreasing temperature. Chemical shifts of proton NMR line vary linearly with decreasing temperature with the slope $d\delta(T)/dT$ linearly depending on sample hydration level. The estimated hydration level for which $d\delta(T)/dT$ equals that for bulk water was $\Delta m/m_0 = 0.267$, which exceeds the hydration level sufficient to initialise ice nucleation in *Cladonia mitis*. The role of biological ice nuclei in promoting the initialization of ice crystallite growth within lichen thallus is discussed. © 2002 Published by Elsevier Science B.V.

Keywords: Proton NMR; Microheterogeneous systems; Bound water; Freezing protection; Lichens; *Cladonia mitis*

1. Introduction

Numerous lichen species may exist in extreme conditions of low temperatures, surviving freezing down to liquid nitrogen temperature [1–4]. Some lichen species can perform the photosynthetic CO_2 uptake at the temperatures below 0 °C and recover such uptake after freezing down to −70 °C independent on freezing rate [1,5–11]. Their photosynthetic activity is maintained for temperatures below the ice heterogeneous nucleation point of lichen thallus fluids [2,3,12,13]. The proteinaceous nature of biological ice nuclei was suggested as they were sensitive on proteases and high concentration of guanidine hydrochloride or urea, whereas there was no effect on chloroform (used as delipidator) [14]. It is not clear whether the ice nucleation in thallus occurs at the same temperature as in cellular fluid in bulk. However,

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in such a case, the lichens may stimulate the growth of ice crystallites in thallus, and as they are dependent on atmospheric moisture, may derive benefit in the form of increased water deposition as a result of ice nucleation. On the other hand, the promotion of freezing at relatively warm temperature lessens frost damage by inducing early formation of smaller, possibly extracellular ice crystals [15]. The unusual adaptative features of lichens make them a very interesting system for the investigation of the resistivity of living systems to low temperature.

Lichen resistance to low temperature is accompanied by the resistance to acute water stress [4]. Lichens passively intake water from the gaseous phase [16–18] and are able to take water from snow omitting the liquid phase [4]. Electrical conduction measurements showed that lichens may reversibly dehydrate down to the two-dimensional percolation threshold of water [19,20] and the clustering point [18,21] which occurs at the hydration level significantly lower than needed to stop living processes in thallus [8,22]. Thus, lichens recover (after rehydration) from the state which (even locally) does not differ from simple amorphic matter. Proton magnetic relaxation experiments show that upon cooling down *Cladonia mitis* Sandst. [= *C. arbuscula* (Wallr.) Flot ssp. Mitis (Sandst.) Ruoss] thallus the reversible transfer of free water to the tightly bound (non-freezable) water pool occurs, which is a freeze protecting process, as non-cooperative ice formation occurs for tightly bound water [23].

Table 1
The scaling series of ^{1}H-NMR spectra for *Cladonia mitis* recorded at $T = 299$ K

Sample	Peak position		Halfwidth	
	Hz	ppm	Hz	ppm
'09'	0	0	150	0.5
	440	1.5	4790	16.0
'76'	490	1.6	1800	6.0
'93'	500	1.7	1180	3.9

The area under peaks was not estimated for this series. For Sample '09' two peaks were recorded.

The aim of our research was to observe the behaviour of loosely bound and free water present in *Cladonia mitis* thallus upon slow (in equilibrium) cooling of the thallus to −45 °C, which is below the environmental temperature range for *Cladonia mitis*. We selected *Cladonia mitis* because it is a cosmopolitic species which occurs in a broad range of climatic conditions. To examine how the effectiveness of protecting mechanism depends on water content, we performed the temperature courses at various hydration level of lichen thallus.

The application of a very soft pulse ($\pi/2 = 35$ μs) allowed us to observe the loosely bound water pool behaviour only. Thus, our study is complementary to the temperature measurement of free induction decays performed for hard pulse ($\pi/2 = 1.1$ μs) [23] and to the proton NMR spectra recorded at intermediate NMR pulse power ($\pi/2 = 8.3$ μs), which selects complete water signal from the total signal intensity of protons in the sample, whereas the solid contribution is significantly reduced [24].

2. Materials and methods

Cladonia mitis was harvested in Northern Sweden, in autumn. The thallus was stored at room temperature in an air-dry state. Prior to NMR experiments, the samples were incubated for 14 days in the atmosphere over the surface of H_3PO_4 ($p/p_0 = 9\%$) . Samples were then placed in NMR tubes, gently pressed, and hydrated for 30 days from gaseous phase over the supersaturated solutions of Na_2SO_4 ($p/p_0 = 93\%$), $Na_2S_2O_3$ (76%), or kept over the H_3PO_4 surface. The obtained Samples '93', '76' and '09' contained $\Delta m/m_0 = 0.193$, 0.126 and 0.076 of water, respectively, where m_0 is dry mass of the sample measured after 48-h incubation in oven at 7 °C.

Proton NMR spectra were recorded on Bruker AM 300 WB spectrometer working at the resonance frequency 300 MHz. The pulse length was $\pi/2 = 35$ μs; the spectral width was 100 kHz. Lorentzian line broadening of 1 Hz was applied for the Sample '09'; all other spectra were processed without any line broadening.

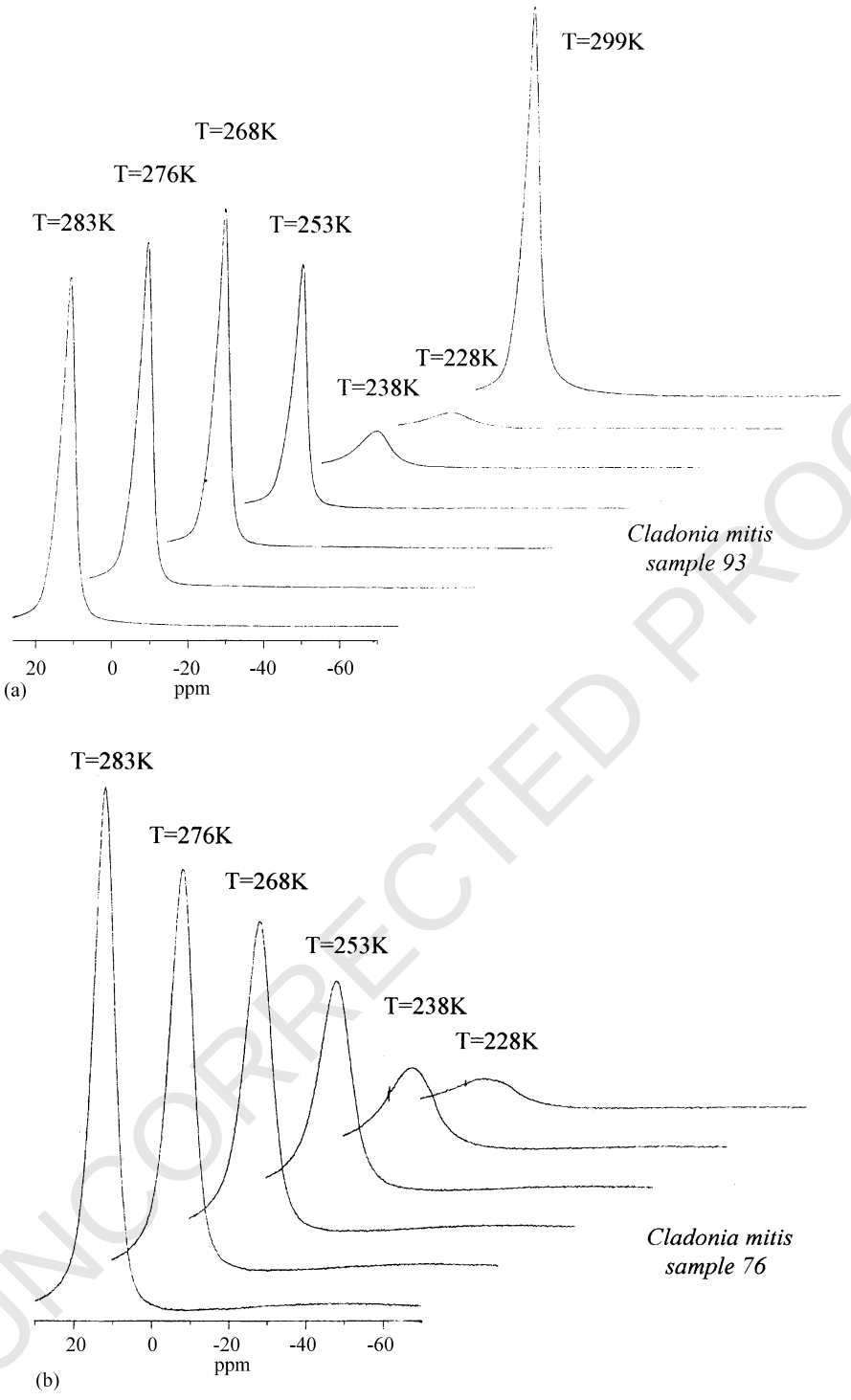

Fig. 1. Proton NMR spectrum recorded at 300 MHz for the thallus of *Cladonia mitis* as a function of temperature: (a) Sample '93' hydrated in $p/p_0 = 93\%$ ($\Delta m/m_0 = 0.193$); (b) Sample '76' hydrated in $p/p_0 = 76\%$ ($\Delta m/m_0 = 0.126$) and (c) Sample '09' hydrated in $p/p_0 = 09\%$ ($\Delta m/m_0 = 0.076$).

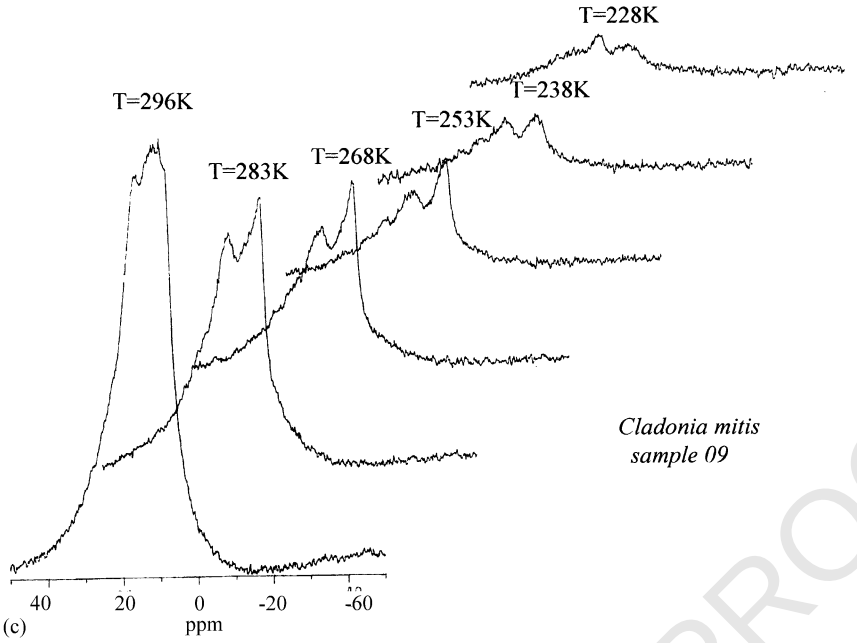

Fig. 1 (*Continued*)

The temperature was stabilized in gaseous nitrogen flow with the accuracy of about 1 K. After the temperature of the stream was reached, there was at least 15 min delay to allow the temperature of the sample to stabilize.

3. Results

3.1. Scaling the spectra

No external reference was used, so, the peak positions of spectra recorded at decreasing temperatures were scaled to the reference points defined in the scaling series of spectra recorded at $T = 299$ K.

As a zero point a narrow peak observed in Sample '09' was taken, which might be assigned to moisture bound on the surface of dry thallus during the sample preparation. It was observable because the overall proton signal of Sample '09' was small. Indeed, after 24 h this signal was no longer observed on the proton spectrum of Sample '09', and it was thought that the water, initially giving rise to the narrow peak, had been adsorbed by thallus after 24 h. This is substantiated by the fact that the hydration level of the thallus stabilized in atmosphere at $p/p_0 = 9\%$ is sufficiently low that exogenous water is very efficiently taken in. Thus, the narrow line observed during the temperature course in the Sample '09' should not be assigned to free external water, but rather to water fraction bound in thallus.

Proton peak positions of the Sample '93' spectra recorded at different temperatures were directly scaled to the peak position recorded at 299 K during scaling series of experiments. For the Sample '76' (for which the temperature course started at 293 K), the peak positions of the spectra of temperature series were scaled to the value extrapolated from the linear least square fitting position of peak (correlation coefficient, $\gamma = -0.9993$). For the Sample '09' peak positions recorded during the temperature course were scaled to the fitted (using least square fits ($\gamma = -0.997$)) position of the broad line central point for the spectrum from scaling series. Line parameters recorded during scaling series are presented in Table 1.

Table 2
^{1}H-NMR spectra vs. decreased temperature recorded for *Cladonia mitis* hydrated at $p/p_0 = 93\%$ (a), at $p/p_0 = 76\%$ (b), and at $p/p_0 = 9\%$ (c)

Temperature		Peak position		Halfwidth		Area
t(°C)	T(K)	Hz	ppm	Hz	ppm	a.u.
a						
26	299	500	1.7	1230	4.1	96.99
10	283	610	2.0	1210	4.0	77.63
3	276	650	2.2	1210	4.0	72.59
−5	268	650	2.2	1140	3.8	70.37
−20	253	680	2.3	1160	3.9	52.93
−35	238	900	3.0	2830	9.4	16.00
−45	228	1000	3.3	4120	13.7	10.26
b						
20	293	540	1.8	1910	6.4	50.41
10	283	630	2.1	2140	7.1	48.48
3	276	680	2.3	2400	8.0	43.02
−5	268	750	2.5	2890	9.6	33.13
−20	253	1000	3.4	4350	14.5	18.04
−35	238	1150±200	3.8±2.6	6290±200	21.0±2.6	12.26
−45	228	1930±150	6.4±0.5	7790±250	26.0±0.8	4.83
c						
23	296	−730	−2.4	1010±250	3.4±0.8	295.45
		1210±250	4.0±0.8	4380±170	14.6±0.6	
10	283	−1240	−-4.1	1010±250	3.4±0.8	205.97
		1460±250	−4.8±0.8	4960±250	16.5±0.8	
−5	268	−980	−3.3	1350±80	4.5±0.3	148.17
		1630±200	5.4±1.4	5050±420	16.8±1.4	
−20	253	−980	−3.3	1350±80	4.5±0.3	104.55
		1880±200	6.2±1.4	6310±670	21.0±2.2	
−35	238	−730±85	−2.6±0.3	1180±170	3.9±0.6	73.20
		1960±420	6.5±1.4	5890±590	19.6±2.0	
−45	228	2260±840	7.5±2.8	8580±840	28.6±2.8	69.68

For Table 2c both peak positions are scaled to the middle of broad peak at room temperature.

The π/2 pulse length equal to 35 μs allowed us to detect the mobile fraction of water, which may consist of losely bound or free water pool. The contribution of tightly bound water signal was significantly reduced, as it relaxes in lichen thallus with $T_2^* = 60$–100 μs and solid signal ($T_2^* \approx 16$ μs, defined as $1/e$ value of Gaussian function) was not observed [18,23].

3.2. Line halfwidth

For the hydrated Samples '93' and '76' proton NMR spectrum recorded at 300 MHz is well described by Lorentzian function in the temperature range investigated. For the Sample '09' the NMR spectrum may be effectively approximated by Lorentzian function only at room temperature ($T = 296$ K), whereas at lower temperature it shows more complex structure with two pronounced peaks (Fig. 1a–c). Fitted peak positions, line widths and of areas under peak are presented in Table 2a–c for the Samples '93', '76' and '09', respectively.

In hydrated samples proton NMR spectrum is an average of free water and of loosely bound water contributions, whereas in dehydrated Sam-

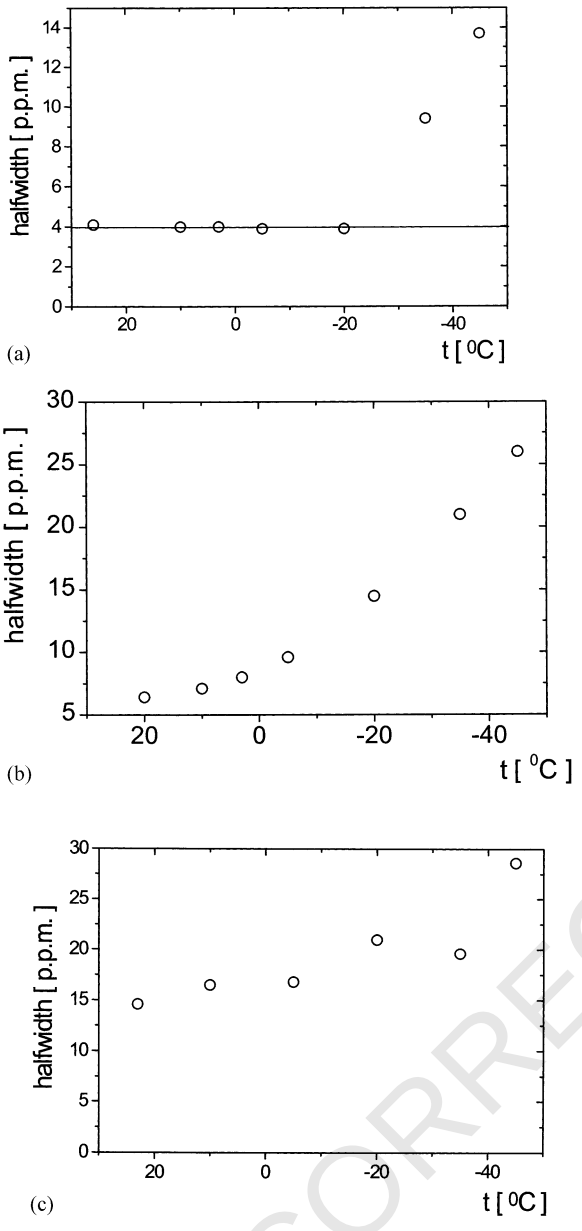

Fig. 2. The temperature dependency of proton NMR linewidth recorded at 300 MHz for the thallus of *Cladonia mitis* hydrated to $\Delta m/m_0 = 0.193$ (a) and to $\Delta m/m_0 = 0.126$ (b).

ple '09' the observed signal is a sum of contributions from mobile protons of thallus and possibly protons from tightly bound water signal partialy excited by the soft NMR pulse.

Fig. 2a and b shows the dependency of the NMR line halfwidth on temperature for *Cladonia mitis* thallus hydrated to $\Delta m/m_0 = 0.193$ and to $\Delta m/m_0 = 0.126$ (Sample '93' and '76', respectively). For the Sample '93' the halfwidth of proton line has a constant value $h_w = (3.98 \pm 0.08)$ ppm ((1190 ± 40) Hz) for temperatures above 253 K. As the sample is solid, gently pressed lichen thallus, which does not form a spatially uniform sample, linewidth measured for the Sample '93' resulted most likely from a distribution of magnetic fields (caused by local changes in susceptibility) experienced by the protons. In contrast, the halfwidth of proton NMR line observed for the Sample '76' continuously increases with decreasing temperature, which, assuming Lorentzian lineshape, gives a linear decrease of spin–spin relaxation time (calculated from the linewidth) on Arrhenius plot with activation energy equal to $E_a = 12.7 \pm 0.4$ kJ mole^{-1}. This value is smaller than that for the formation/breaking of hydrogen bonds in liquid water. However, most likely this value is altered by the influence of paramagnetic ions present in aqueous medium in thallus.

3.3. Peak positions

Peak positions of NMR lines for *Cladonia mitis* thallus at different hydration levels are presented in Fig. 3. For all the samples, proton NMR peak position for *Cladonia mitis* shifts upwards with decreasing temperature, however, the slope of this function depends on hydration level of the sample. The chemical shift is a measure of the change in resonant magnetic field strength when the environment of a proton changes [25]. If an O–H group of a water molecule forms a hydrogen bond the magnetic resonant field strength decreases and the signal shifts downfield.

The temperature dependence of the chemical shift, δ, found for liquid water is expressed by phenomenological formula [26,27]:

$$\delta = -4.58 + 0.0095t \quad (1)$$

where temperature t is expressed in Celsius scale. The signal shifts downfield as steam is condensed and shifts further downfield as water is cooled. Similar shifts are observed for other substances

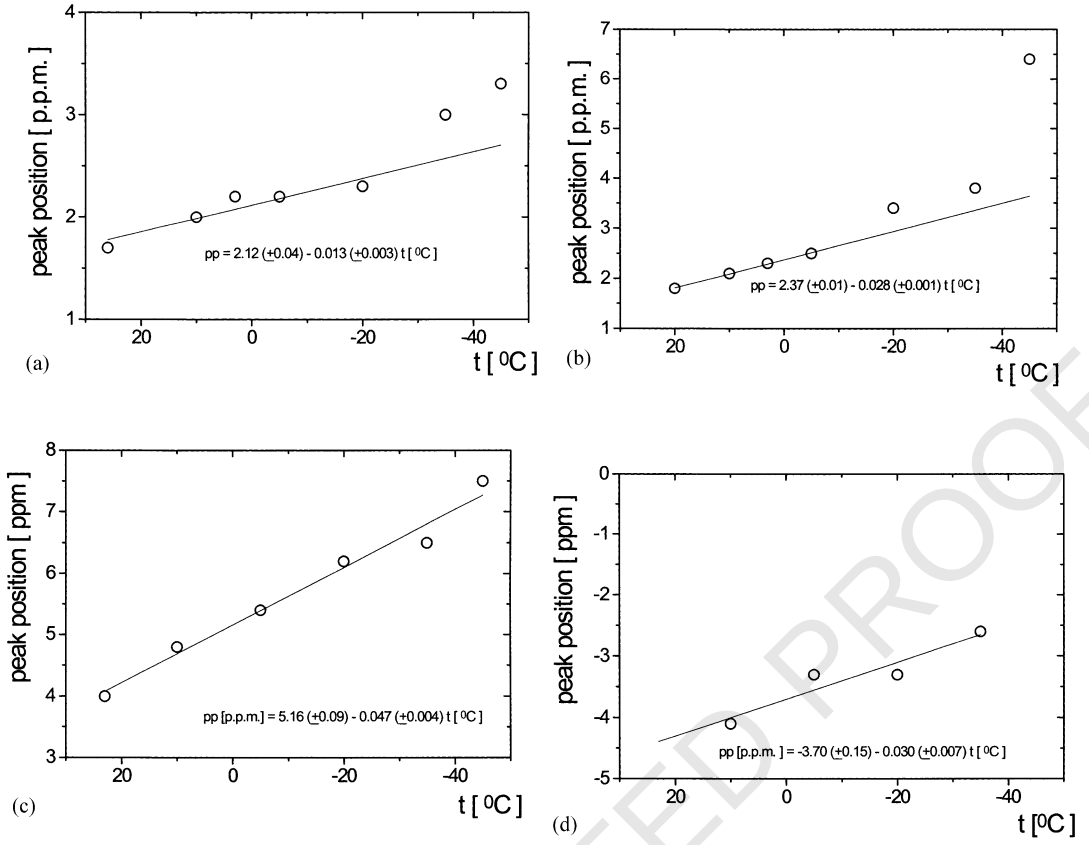

Fig. 3. The temperature dependency of the proton NMR line peak position recorded at 300 MHz for the thallus of *Cladonia mitis* hydrated to $\Delta m/m_0 = 0.193$ (a) and to $\Delta m/m_0 = 0.126$ (b). In the sample hydrated to $\Delta m/m_0 = 0.076$ a broad peak was observed (c) and a narrow one (d). Straight lines were fitted to the data recorded above -20 °C, whereas for the Sample '09' to all recorded data. To emphasize the discrepancy caused by water immobilization, the fitted lines are extended to lower temperature for all samples.

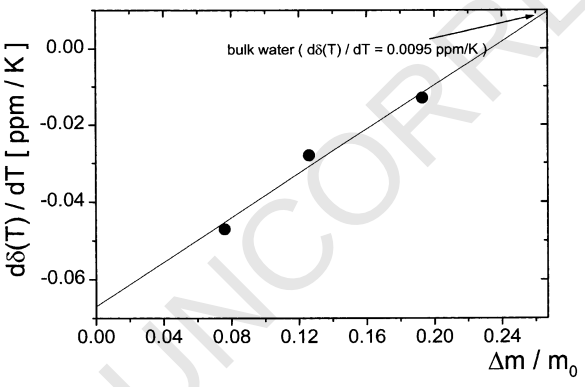

Fig. 4. The slope of the proton chemical shift temperature dependency, $d\delta(T)/dT$, for *Cladonia mitis* thallus hydrated to different levels ($\Delta m/m_0$).

forming hydrogen bonds. Usually shift is larger as hydrogen bond formed is stronger [25]. When O–H group enters into a hydrogen bond, the electronic environment of the proton changes in such a way that the screening constant, σ is reduced. Thus, the local magnetic field acting on the hydrogen bond proton, B_{loc} increases according to:

$$B_{loc} = B_0(1 - \sigma) \quad (2)$$

where B_0 is applied magnetic field, and the resonance is observed at lower value of the applied magnetic field. This effect occurs because (a) the presence of oxygen O_B in the $O_A - H \cdots O_B$ hydrogen bond changes the distribution of electronic charge in the O_A–H bond in such a way that it

tends to draw the proton away from the electrons in the O_A–H bond, and reduces the electron density around the proton (resulting in reduction of σ and thus causing a chemical shift downfield); or (b) induced electron currents in O_B produce a magnetic field at the proton. Effect (b) is significant only if the magnetic susceptibility of O_B is anisotropic and can alter σ value [28].

The dependency of the chemical shift of water on temperature has been interpreted both in terms of hydrogen-bond breaking and hydrogen-bond distortion. The interpretation in terms of bond breaking [27,29] is based on the assumption that the chemical shift observed at given temperature, $\delta(T)$, is an average of the chemical shifts of hydrogen-bonded and non-hydrogen-bonded protons in the liquid (δ_{HB} and $\delta_{n\text{-}HB}$, respectively). In this model the observed chemical shift may be written:

$$\delta(T) = X_{HB}(T)\delta_{HB} + [1 - X_{HB}(T)]\delta_{n\text{-}HB} \quad (3)$$

where $X_{HB}(T)$ is the mole fraction of intact hydrogen bonds at temperature T. As $X_{HB}(T)$ decreases with increasing temperature [30] $\delta(T)$ moves upfield with increasing temperature.

Muller and Reiter [31] showed that the temperature dependency of the chemical shift of hydrogen bonded substances may in part arise from distortion (the stretching) of hydrogen bonds. Hindmann [29] emphasized that the stretching and bending of hydrogen bonds can probably contribute to the chemical shift of water.

Fig. 3 shows the chemical shift for protons of water in thallus of *Cladonia mitis* at various hydration levels. For the temperature range below 0 °C, chemical shift shifts upfield with decreasing temperature. For a given hydration level the value $d\delta(T)/dT$ remains constant as the temperature is decreased, except for the Sample '93' hydrated to $\Delta m/m_0 = 0.193$, for which a rapid decrease in the area under peak (accompanied by the increase in the line halfwidths) occurs between −20 and −35 °C. This is caused by the freezing of the loosely bound water fraction. The spectrum of the Sample '09' is a sum of two lines: a broad line and a narrow one. The linewidth of the narrow line is close to the one recorded for the Sample '76' with the value $d\delta(T)/dT$ almost equal to the value for the Sample '76'. We suggest that the narrow signal

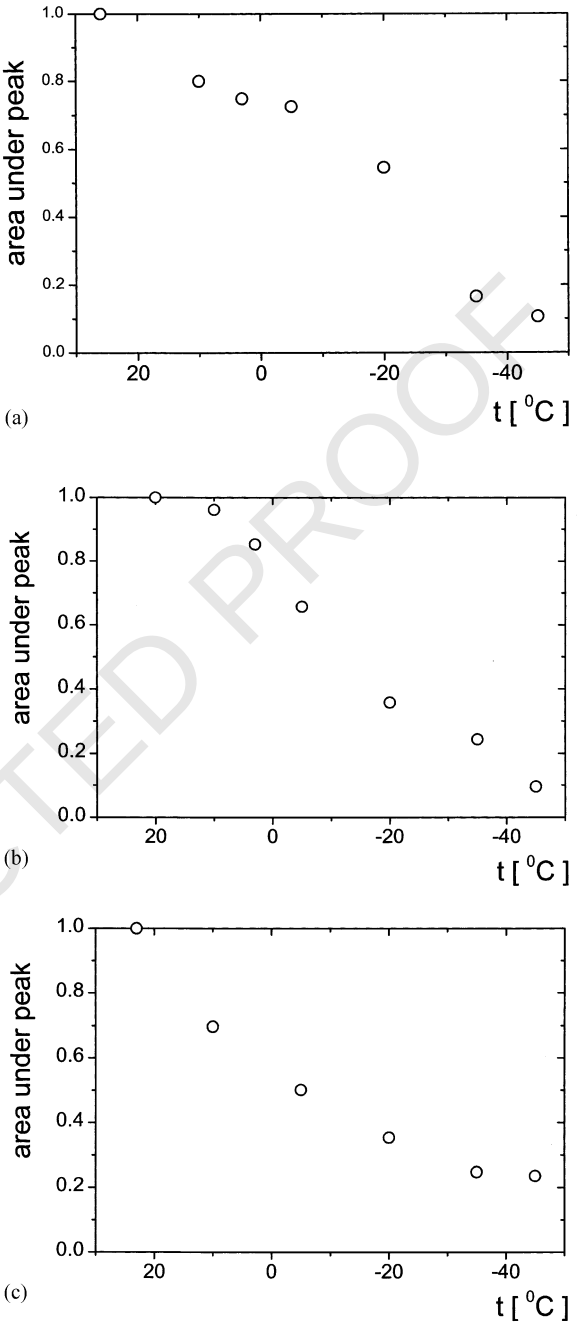

Fig. 5. The temperature dependency of the proton NMR line area recorded at 300 MHz for the *Cladonia mitis* thallus hydrated to $\Delta m/m_0 = 0.193$ (a), to $\Delta m/m_0 = 0.126$ (b) and to $\Delta m/m_0 = 0.076$ (c).

may come from water fraction which reveals similar behaviour to loosely bound water present in more hydrated Sample '76'. Such a signal may come from isolated compartments containing remnants of water fraction dominating in more hydrated sample, whereas the vast majority of water is in structural state characteristic for lower hydration level. Such a narrow peak was not observed in other series of experiments performed on other *Cladonia mitis* sample collected at the same site [24].

The opposite tendency of chemical shift temperature dependence may be caused by the non-cooperative immobilization of water molecules: as they bound to inner surfaces of thallus, the average number of hydrogen bonds between the molecules in liquid water decreases; as the layer of water loosely bond to the surface is relatively thin, also the relative number of hydrogen bonds between mobile water molecules decreases. If so, the $d\delta(T)/dT$ value should depend on the hydration level of the sample. Fig. 4 presents the slope of the chemical shift temperature dependence, $d\delta(T)/dT$, as a function of hydration level. For hydrated samples and for broad peak of the Sample '09' the value of $d\delta(T)/dT$ linearly depends on hydration level. The linear change suggests the contribution of two components: from water in bulk and from water in contact with the inner surface of thallus. As hydration level, at low water amount, is a linear function of the thickness of water layer, we may expect linear form of the dependency.

The linear function:

$$\frac{d\delta(T)}{dT} = -0.067(\pm 0.006) + 0.287\left(\pm 0.044\frac{\Delta m}{m_0}\right) \quad (4)$$

fits the data with the correlation coefficient equals to $\gamma = 0.989$.

If this is the case, from Eq. (4) one can get the hydration level needed for function $d\delta(T)/dT$ to reach the value for bulk water (Eq. (1)). Free water limit is reached for $\Delta m/m_0 = 0.267$, which, as estimated from proton relaxation data [23], is very close to the maximum hydration level ($\Delta m/m_0 = 0.260$) for *Cladonia mitis* thallus below which formation of ice crystallites does not occur.

3.4. Area under peaks

For *Cladonia mitis* thallus at all investigated hydration levels, the area under proton NMR line decreases with decreasing temperature. For the sample hydrated to $\Delta m/m_0 = 0.193$ (Fig. 5a), at temperatures above $t = -20$ °C the decrease is continuous, but for the spectra recorded at the lowest temperatures area under peak decreases to approximately 0.1 of the value at the temperature $t = 26$ °C. The jump in area under peak at about $t = -20$ °C is correlated with the rapid increase in proton linewidth (Fig. 2a) and reflects both the freezing of significant water fraction and the dramatic change in mobility of the remaining non-frozen water pool.

Fig. 5b shows the temperature dependence of the area under proton NMR peak for *Cladonia mitis* thallus hydrated to $\Delta m/m_0 = 0.126$. As at higher hydration level, the area under peak decreases with decreasing temperature, down to the value of 0.096 of the value at $t = 20$ °C. The decrease is continuous and there is no pronounced jump in area-under-peak temperature dependency. The decrease of more than 90% water signal is caused by freezing protection mechanism of water transfer from loosely to tightly bound water pool as it was previously detected [23]. As power of NMR pulse was not sufficient to record the complete tightly bound water signal in *Cladonia mitis*, we could not separate the decrease of proton signal caused by ice nucleation and the contribution of the transfer mechanism of water from loosely bound to tightly bound water fraction (see also Ref. [24]).

The temperature dependence of the area under proton NMR line in *Cladonia mitis* hydrated to $\Delta m/m_0 = 0.076$ is shown in Fig. 5c. For this sample the proton NMR signal decreases smoothly with decreasing temperature and at $t = -45$ °C reaches 0.236 of the value recorded at $t = 23$ °C. Like in Sample '76', the decrease in area under peak with decreasing temperature is smooth. NMR line for the dehydrated thallus is significantly broader than the ones for samples hydrated to higher level. The

amount of free water spread over the inner thallus surfaces in dehydrated *Cladonia mitis* is not sufficient to dominate in the NMR signal (and to activate the freezing protection transfer mechanism), thus, the more tightly bound water fraction present in the thallus is observed.

4. Discussion

The conformational change occurring at $t = -30$ °C in dehydrated *Cladonia mitis* ($\Delta m/m_0 = 0.046$) observed by proton relaxometry using hard pulses [32] did not manifest itself in either peak position or peak halfwidth in the present experiments in dehydrated sample. Also the area under peak changes smoothly with decreasing temperature. This confirms the conclusion that the transition at −30 °C is caused by structural changes in lipids of *Cladonia mitis* membranes, and it is not seen by water bound on membrane surface.

Freeze protection mechanism in lichen manifests itself in the recovery of the CO_2 uptake after cooling the thallus down and in photosynthetic CO_2 uptake at low temperatures. Variety of published data provide convincing evidence that there exists an extraordinarily effective freezing protection mechanism in thallus enabling it to recover the living activity even after freezing in liquid nitrogen. However, there is still very little known about the molecular mechanisms of thallus frost protection.

To explain the freeze protection mechanism the cryoprotective action of sugars and polyols was suggested, as lichen mycobionts may effectively (up to 15% w/w) deposit monosaccharides and polyols [33]. Polyols act as a cryoprotective agent in thylakoid membranes [34,35]. The cryoprotective action of polyols is based on blocking the formation of ice crystallites by steric mismatch of hydrogen bonds, which may be formed between them and water. One may suppose that also in lichens sugars and polyols may play a significant role in the frost protection mechanism. However, this is only partially responsible for the phenomena observed by proton NMR. We did not observe the increase in the liquid signal, as should occur in the case of intensive production of liquid polyols by dissolving the solid matrix of the thallus (such an effect is seen as water-soluble fraction of horse-chestnut bast is dissolved at mild hydration of bast [36]). In contrast, the total liquid signal decreased with decreasing temperature. The proton relaxation [23] as well as proton spectra recorded by us clearly suggest that during cooling the thallus down the amount of tightly bound (non-freezable) water increases very significantly. This may be achieved by formation of a 'gel-like' structure filling the volume of cellular fluids.

In the Sample '93' at temperature $t = -20$ °C the discontinuous decrease of the area under peak accompanied by the increase of the line halfwidths occurs, which is caused by the freezing of the loosely bound water fraction. The temperature of transition was lower as the one reported elsewhere [23]. It is possible that the temperature of thallus could be a little higher than the temperature recorded by sensor, because the air present in the sample tube may insulate pieces of thallus. For both Samples '76' and '09' the thermal changes of area under peak, peak halfwidths and the peak position vary continuously with decreasing temperature. This means that the cooperative freezing was not observed in samples hydrated to $\Delta m/m_0 = 0.126$ or less. Cooperative freezing observed in the sample hydrated to $\Delta m/m_0 = 0.193$, occurs for hydration level lower then the value obtained from linear estimation of the freezing protection mechanism effectiveness ($\Delta m/m_0 = 0.26$ [23]). Also the estimation of the proton peak position temperature dependency showed that the free water limit is reached for the hydration level higher than the one at which the ice crystallite formation in *Cladonia mitis* thallus occurs. On the other hand, in the lichen thallus fluids the ice nucleation takes place [2,12,13] for the temperatures well above the lower limit of photosynthetic activity. If it is so in lichen thallus, the ice nucleation might not be a destructive process in this material (or its destructive effects are somehow compensated). The ice crystallite formation may be promoted by the presence of proteinaceous nuclei [14].

Acknowledgements

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