Freezing of water bound in lichen thallus as observed by $^1$H NMR. II. Freezing protection mechanisms in a cosmopolitan lichen *Cladonia mitis* and in Antarctic lichen species at different hydration levels

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Abstract

Proton NMR spectra at 300 MHz for dehydrated and hydrated thallus of *Cladonia mitis* Sandst. [= *C. arbuscula* (Wallr.) Flot ssp. Mitis (Sandst.) Ruoss], *Himantormia lugubris* (Hue) Lamb and *Usnea aurantiaco-atra* (Jacq.) Bory were recorded. The temperature was decreased from room temperature down to $-45^\circ$C. Pulse length was set to $\pi/2 = 8.3$ µs, which allowed the observation of tightly bound and loosely bound/free water fraction, whereas the signal from solid matrix of thallus was reduced. In hydrated thallus a narrow Lorentzian line coming from loosely bound/free water fraction was recorded. For the temperature range between $-5$ and $-20^\circ$C a discontinuous increase in line halfwidths, accompanied by a decrease in area under the peak, was observed. This was attributed to the cooperative freezing of bulk water present in lichen thallus. In dehydrated thallus the NMR line consists of two components: a narrow, Lorentzian one (coming from loosely bound/free water fraction) and a broad line (from water tightly bound in lichen thallus). The overall area under peak remains unchanged down to $-5^\circ$C, and then between $-5$ and $-20^\circ$C it continuously decreases due to non-cooperative water immobilisation. As the temperature is decreased, for temperatures above $-5^\circ$C, the contribution made by the broad line component increases at the expense of the narrow line component. The mechanism of loosely-to-tightly bound water transfer is, at least partially, responsible for the freeze-protection of thallus in the lichen species investigated. No significant differences between the freeze protecting loosely-to-tightly bound water transfer mechanism of Antarctic lichens and that of cosmopolitan lichens was noticed. © 2002 Published by Elsevier Science B.V.

Keywords: Proton NMR; Microheterogeneous systems; Freezing protection; Lichens; *Cladonia mitis*; *Himantormia lugubris*; *Usnea aurantiaco-atra*

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1. Introduction

Several species of lichens are well adapted to function at extremely low temperature and low hydration level [1]. They recover the life activity after low temperature stress (for some species after the temperature of liquid nitrogen) [2–5] and perform their living functions (the photosynthetic CO₂ uptake) below the water freezing point [1,6–10] and even below the ice heterogeneous nucleation point of lichen thallus fluids [4,11,12].

Lichens are able to take water from snow omitting the liquid phase and the hydration level obtained in this way is sufficiently high to start the photosynthetic activity [5]. They passively take water from the gaseous phase [13–15]. Himantormia lugubris and Cladonia mitis reversibly dehydrate down to the two-dimensional percolation threshold of water [16,17] and water clustering point [15] which occurs at hydration level lower than needed to stop living processes in thallus [8,18].

The aim of our research was to observe the behaviour of tightly and loosely bound water during slow (in equilibrium) cooling of the thallus from room temperature down to −45 °C. We selected three lichen species; two Antarctic samples: Himantormia lugubris, Usnea aurantiaco-atra and a cosmopolitan one: Cladonia mitis. To examine the dependence of the effectiveness of freeze-protection mechanism on water content, we performed the temperature courses at two hydration levels of thallus, both obtained through hydration from the gaseous phase.

Proton spectra were recorded using the pulse power sufficient to observe both tightly bound water and free water signals (π/2 = 8.3 μs), whereas the signal from solid matrix of thallus was significantly reduced. Thus, our study is complementary to the temperature dependent measurements in the time domain (free induction decays) using hard pulses (π/2 = 1.1 μs) [19], and to the experiment involving soft pulses (π/2 = 35 μs) [20], where solid signal was not observed and the signal from tightly bound water was reduced.

2. Materials and methods

Himantormia lugubris and Usnea aurantiaco-atra were collected in King George Island, South Shetlands during the Antarctic summer, whereas Cladonia mitis was collected in Northern Sweden, in autumn. The thallus was stored at room temperature and room humidity.

The vitality tests using the fluoresceine biacetate and etidine bromide showed that in Cladonia mitis thallus 50–60% of cells reacted positively, in Himantormia lugubris 40–50% of cells and in Usnea aurantiaco-atra 80%.

Prior to NMR experiments, each type of thallus was divided into two samples. In each case, one was incubated for 48 h over the surface of H₃PO₄ (pH₀ = 9%) and the other over a supersaturated solution of Na₂SO₄ (93%). Samples were then placed in NMR tubes and gently pressed. NMR tubes were sealed using plastic tube sealings (American Can Company). Tight caps prevent the samples from the change of moisture content during the experiments.

Proton NMR spectra were recorded on a Bruker AM 300 WB spectrometer working at the resonance frequency of 300 MHz. The pulse length was π/2 = 8.3 μs. The number of scans was 4 for the hydrated sample and 16 for the dry one. The relaxation delay was 3s between scans. The number of data points recorded per spectrum was 4 K; all spectra were processed without line broadening. After averaging the RSS value was between 1 and 1.6 per data point.

The temperature was stabilised in gaseous nitrogen flow with the accuracy of about 1 K. Line deconvolutions were performed using about 1200 data points per spectrum. Based on simplex procedure, the Bruker LINESIM program was used. For dehydrated samples, several different functions were tested to fit the spectrum properly; namely, the superposition of Lorentzian and Gaussian line, two Lorentzians and two Gaussians. The narrow line component was well fitted using Lorentzian line in all samples investigated, whereas the broad line is fitted with sufficiently good quality using either Gaussian or Lorentzian function. This agrees with measurements in time-domain, where tightly bound water...
The signal is fitted by an exponential function [15,19]. The fits assuming individual Lorentzian line shape were good (RSS value between 1 and 1.6 per data point).

3. Results

As the solid matrix of the thallus relaxes with $T_2^*$ $\approx$ 16 $\mu$s (time after which Gaussian signal decreases to its 1/e value) and the tightly bound water fraction relaxes exponentially with $T_2^*$ = 60–100 $\mu$s [15], the $\pi/2$ pulse length of 8.3 $\mu$s allowed us to detect both mobile and tightly bound fraction of water bound to the lichen thallus, whereas the signal coming from the solid matrix of thallus was significantly reduced.

The spectra of dehydrated thalli consisted of two line components: broad and narrow. For hydrated samples the broad line component was hardly visible and only Cladonia mitis revealed the pronounced contribution of broad line (Fig. 1). Fitted line widths and areas under peak are presented in Table 1, Table 2, and Table 3 for Cladonia mitis, Himantormia lugubris, and Usnea aurantiaco-astra, respectively.

The narrow line component may be connected with the free water or water loosely bound in the lichen thallus. The halfwidths of the broad line component was about 32–39 kHz. Except for

Fig. 1. Proton NMR spectrum recorded at 300 MHz for the thallus as a function of temperature; (a) Cladonia mitis, the sample hydrated in $p/p_0 = 93\%$, upper plot, the sample hydrated in $p/p_0 = 9\%$, lower plot; (b) Himantormia lugubris, the sample hydrated in $p/p_0 = 93\%$, upper plot, the sample hydrated in $p/p_0 = 9\%$, lower plot; (c) Usnea aurantiaco-astra, the sample hydrated in $p/p_0 = 93\%$, upper plot, the sample hydrated in $p/p_0 = 9\%$, lower plot.
Cladonia mitis, it was not possible to deconvolute the composite spectrum for hydrated sample, because the contribution of loosely bound/free water pool overwhelms the tightly bound water fraction. For sake of consistency, we present the total areas under peaks and narrow peak positions.

Fig. 2a, c, and d shows the halfwidths of the proton line for hydrated samples of investigated lichens. For the temperature range above $-20 \, ^\circ C$ the line halfwidth is constant and equals 6.78 ppm for Cladonia mitis, 5.39 ppm for Himantoria lugubris and 5.20 ppm for Usnea aurantiaco-astra. As the sample is macro-heterogeneous, the measured value of linewidth is significantly broadened both by the non-uniformity of the effective magnetic field, $B_{\text{eff}}$, and by the magnetic susceptibility non-uniformity. For lower temperature the proton line broadens. This is attributed to freezing of the loosely bound water fraction at $-12 \, ^\circ C$. This interpretation is consistent with high power proton relaxometry results in Cladonia mitis [19,21].

For hydrated Cladonia mitis the spectrum may be deconvoluted into the sum of two components (Fig. 2b). For the temperatures above $-20 \, ^\circ C$ the average Lorentzian line halfwidth ($6.45 \pm 0.24$ ppm) obtained from the deconvolutions is only slightly different from the corresponding value of the linewidth (Fig. 2a) obtained in the single component analysis. The broad Gaussian line is observed with the halfwidth of about 100 ppm for the whole range of investigated temperatures. For the temperatures below $-20 \, ^\circ C$ it significantly broadens, which means that the structural change in loosely bound water fraction affects also the tightly bound water protons (Fig. 2b).

For hydrated samples of lichen thalli the total area under peak remains constant (or slightly decreases) with decreasing temperature down to $t = -10 \, ^\circ C$ (Fig. 3), whereas, for further decreas-
ing temperature the total area under peak rapidly decreases. At $t = -40 \, ^\circ C$ the total area under line is 50% of its initial value. The decrease in area under line is correlated with the line halfwidth increase, suggesting that the free water pool at low temperatures may be transferred to a phase which differs from the free water observed at higher temperature.

Fig. 4 shows the temperature dependence of the area under broad and narrow component of the proton NMR line for the dehydrated thallus of the investigated lichens. For *Himantormia lugubris* and for *Usnea aurantiaco-atra*, the total signal remains almost constant for temperatures above $t = -10 \, ^\circ C$, whereas for lower temperatures it smoothly decreases with decreasing temperatures. For *Cladonia mitis* the signal continuously decreases with decreasing temperature for the whole temperature range, which is the effect implied by the desorption isotherm requirements [22].

The behaviour with temperature of the tightly bound and loosely bound water signal intensities are found to be similar for Antarctic and cosmopolitan lichen species. As the temperature is decreased, the loosely bound water signal significantly decreases. This decrease is compensated for by the increase in the tightly bound water signal. The decrease in mobile line component is almost linear with temperature for *Himantormia lugubris* and for *Usnea aurantiaco-atra*, whereas for *Cladonia mitis* its magnitude stabilises at 8.7% of the total signal for the temperature $t = -20 \, ^\circ C$ and lower temperatures. None of the samples show any discontinuities or breaks in the slope of temperature dependence of area under peak at $t = -35 \, ^\circ C$.

Our results suggest that the process of the transfer of loosely water signal to the tightly bound water pool above $t = -10 \, ^\circ C$ occurs continuously with the temperature decrease.

### 4. Discussion

In hydrated thalli, below $-10 \, ^\circ C$ a rapid decrease in the total area under peak was observed, which is caused by the freezing of loosely bound water. This discontinuity was observed at...
Table 1
The temperature course for Cladonia mitis hydrated at \( p/p_0 = 9\% \) (a) and at \( p/p_0 = 93\% \) (b)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Area under peak</th>
<th>Temperature</th>
<th>Halfwidth</th>
<th>Area under peak (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t ) (°C)</td>
<td>( T ) (K)</td>
<td>(a.u.)</td>
<td>Broad (%)</td>
<td>Narrow (%)</td>
</tr>
<tr>
<td>24</td>
<td>297</td>
<td>227.4</td>
<td>61.5</td>
<td>38.5</td>
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<tr>
<td>10</td>
<td>283</td>
<td>215.5</td>
<td>77.2</td>
<td>22.8</td>
</tr>
<tr>
<td>3</td>
<td>276</td>
<td>203.2</td>
<td>78.6</td>
<td>21.4</td>
</tr>
<tr>
<td>-5</td>
<td>268</td>
<td>184.2</td>
<td>82.4</td>
<td>17.6</td>
</tr>
<tr>
<td>-20</td>
<td>253</td>
<td>152.8</td>
<td>90.0</td>
<td>10.0</td>
</tr>
<tr>
<td>-35</td>
<td>238</td>
<td>104.2</td>
<td>91.8</td>
<td>8.8</td>
</tr>
<tr>
<td>-45</td>
<td>228</td>
<td>82.3</td>
<td>92.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

\(-12 \, ^\circ C\) by high power proton relaxometry in the thallus of Cladonia mitis [19,21].

Table 2
The temperature course for Himantormia lugubris hydrated at \( p/p_0 = 9\% \) (a) and at \( p/p_0 = 93\% \) (b)

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Area under peak</th>
<th>Temperature</th>
<th>Halfwidth</th>
<th>Area under peak (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( t ) (°C)</td>
<td>( T ) (K)</td>
<td>(a.u.)</td>
<td>Broad (%)</td>
<td>Narrow (%)</td>
</tr>
<tr>
<td>24</td>
<td>297</td>
<td>224.53</td>
<td>70.0</td>
<td>30.0</td>
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<tr>
<td>10</td>
<td>283</td>
<td>226.20</td>
<td>75.0</td>
<td>25.0</td>
</tr>
<tr>
<td>3</td>
<td>276</td>
<td>219.88</td>
<td>82.3</td>
<td>17.7</td>
</tr>
<tr>
<td>-5</td>
<td>268</td>
<td>199.92</td>
<td>82.7</td>
<td>17.3</td>
</tr>
<tr>
<td>-20</td>
<td>253</td>
<td>168.49</td>
<td>90.4</td>
<td>9.6</td>
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<td>238</td>
<td>121.95</td>
<td>97.3</td>
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<td>-45</td>
<td>228</td>
<td>111.47</td>
<td>99.2</td>
<td>0.8</td>
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</table>

\(-12 \, ^\circ C\) by high power proton relaxometry in the thallus of Himantormia lugubris. A break in the slope of the temperature dependence of proton spin-lattice relaxation time at \( t = 238 \).
−35 °C was observed in the dehydrated thallus of Cladonia mitis [21]. As this effect was observed only in dehydrated samples, it suggested that it is caused by the structural changes in the thallus solid matrix. The present experiments did not reveal any discontinuities in the area under peak at \( t = −35 °C \) for dehydrated thallus. As these experiments did not record the signal from solid matrix of thallus, but from bound water only, the above supports the hypothesis that the effect at \( t = −35 °C \) occurs in solid matrix of lichen thallus and, moreover, the tightly bound water layer is not involved in this process [21].

The change in the slope, at \( t = −45 °C \), of the proton spin-lattice relaxation time versus temperature plot was attributed to subtle structural change in water tightly bound in thallus of Cladonia mitis [21]. This effect was not observed here as the present measurements were not performed below \( t = −45 °C \).

The variety of data available on the ability of lichen to recover CO₂ uptake after cooling the thallus down to low temperatures, taken together with direct recording of photosynthetic CO₂ uptake at low temperatures represents convincing evidence that an extraordinarily effective freezing protection mechanism exists in the thallus. This mechanism enables it to recover living activity even after freezing in liquid nitrogen and must preserve the thallus during ice crystallite growth which otherwise might destroy thallus microstructures. The physical basis for such a freezing protection mechanism has not been established so far. However, observations of Antarctic arthropodes and variety of plants [23–29] suggest that polyhydric alcohols and simple sugars may act as cryoprotectants. Glycerol present in aqueous medium of wheat photosynthetic membrane preparations protects the system from the ice crystallite formation [30,31]. Although glucose and fructose were not found in Evernia esoredisa (Müll. Arg.) Du Rietz, in Ramalina subbreviuscula Asah., and in R. sblitoralis Ash., in samples collected in nature, and the significant content of monosugars was only forced by external osmotic conditions, the polyols (ribitol, mannitol, arabitol), as essential metabolite, were present in thalli on constant level (up to 3.4% w/w of arabitol in R. subbreviuscula) [32] and may act as cryoprotective agents in thallus. Using \(^{13}\)C-NMR, Chapman et al.

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**Table 3**

The temperature course for *Usnea aurantiaco-atra* hydrated at \( \rho/\rho_0 = 9\% \) (a) and at \( \rho/\rho_0 = 93\% \) (b)

<table>
<thead>
<tr>
<th>( t (°C) )</th>
<th>( T (K) )</th>
<th>( T (a.u.) )</th>
<th>Broad (%)</th>
<th>Narrow (%)</th>
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<td>195.56</td>
<td>64.2</td>
<td>35.8</td>
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<tr>
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<td>283</td>
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<td>23.8</td>
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<tr>
<td>3</td>
<td>276</td>
<td>189.54</td>
<td>79.6</td>
<td>20.4</td>
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<tr>
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<td>268</td>
<td>180.91</td>
<td>84.3</td>
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<td>−45</td>
<td>228</td>
<td>93.92</td>
<td>99.1</td>
<td>0.9</td>
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</table>

<table>
<thead>
<tr>
<th>( t (°C) )</th>
<th>( T (K) )</th>
<th>( (\text{Hz}) )</th>
<th>(ppm)</th>
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<tbody>
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<td>297</td>
<td>1606.0</td>
<td>5.35</td>
<td>145.32</td>
</tr>
<tr>
<td>10</td>
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<td>144.88</td>
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<td>1516.7</td>
<td>5.05</td>
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<td>−5</td>
<td>268</td>
<td>1517.6</td>
<td>5.05</td>
<td>144.42</td>
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<tr>
<td>−20</td>
<td>253</td>
<td>1625.1</td>
<td>5.41</td>
<td>132.21</td>
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<td>−35</td>
<td>238</td>
<td>2515.4</td>
<td>8.38</td>
<td>97.75</td>
</tr>
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<td>−45</td>
<td>228</td>
<td>4024.2</td>
<td>13.4</td>
<td>79.34</td>
</tr>
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</table>
[33] identified polyols and sugars, and quantified their abundance, in several Antarctic lichens. Polyol content varied between 17 mg g\(^{-1}\) for Candelariella hallettenensis (Murray) Ørsted [= C. flava (Dodge & Baker) Castello & Nimis] and 65 mg g\(^{-1}\) for Usnea antarctica Du Rietz, with dominating contribution from arabitol, mannitol and ribitol, whereas sorbitol was not detected. Sugar content was approximately one order of magnitude smaller than the content of polyols. In Antarctic bryophytes the repeated freeze-thaw cycles (up to \(n = 16\)) increase rates of carbohydrate...
loss, which is not significantly correlated with the
DSC-detected freezing temperature of tissue [34].

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. Although the
overall concentration of polyols detected in the
thallus is not sufficient to promote significant
decrease of freezing point of cellular aqueous
medium, if they are localized mainly in intracel-
lar spaces, they may contribute to the frost
protection mechanism in lichens.

We did not observe the increase of the total
liquid proton signal upon cooling the thallus, as it
should be observed if liquid polyols were rapidly
produced by dissolution of the solid matrix of dry
biological system [35]. We propose that the
mechanism of water transfer from loosely bound
(freezable) to tightly bound (non-freezable) pool
may play a significant role in freeze protection of
lichen thallus. The tightly bound water may
produce an ‘insulating’ layer surrounding the ice
crystallites as they are probably formed in inner
spaces of lichen thallus.

In lichen cellular fluids ice nucleation is ob-
served [3,11,12,36,37] at temperatures well above
the lowest temperature at which the photosyn-
thetic activity occurs. If this is the case for the
lichen thallus, ice nucleation in this system is not
necessarily a destructive process.

It is thought that extracellular water freezing
occurs in freezing tolerant plants and the lethal
process of intracellular formation of hexagonal ice
crystal is avoided [38]. Most likely, in lichen, the
thallus acts not only to protect it from ice crystal-
lite growth in intra-cellular spaces during freezing,
which otherwise might destroy the thallus micro-
structure, but also to promote ice crystallite
formation in extra-cellular spaces, which mini-
mizes the effect of potentially destructive intra-
cellular ice crystallite formation. The effect of the
stimulation of the non-lethal growth of ice crystal-
lites in extra-cellular space may result in accumu-
lation of the increased water amount in form of ice
crystallites (in extra-cellular space), which can be
advantageous for lichens living in sites with
permanent moisture deficit.

Acknowledgements

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