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Protein adaptation to low temperatures: a comparative study of α-tubulin sequences in mesophilic and psychrophilic algae

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Abstract The α-tubulin genes from two psychrophilic algae belonging to the genus *Chloromonas* (here named ANT1 and ANT3) have been isolated and sequenced. The genes ant1 and ant3 contain 4 and 2 introns, respectively. The coding DNA sequences are 90% identical but the degree of isology is very high at the polypeptide level (more than 97% strict identities). The ANT1 and ANT3 α-tubulin amino acid sequences were compared to the corresponding sequence of the mesophilic alga *Chlamydomonas reinhardtii*. Of the 15 substitutions detected in ANT1 and/or ANT3, 5 are common to both psychrophilic algae. The recorded substitutions have been analyzed in terms of cold adaptation on the basis of the available three-dimensional structure of the α,β-tubulin heterodimer from pig brain. Most of these are subtle changes, but two substitutions, M268V and A295V occurring in the region of interdimer contacts, could be of great significance for the cold stability of Antarctic algae microtubules due to the fact that the entropic control of microtubule assembly is particularly high in cold adaptes species.

Key words Tubulin · Antarctic algae · Cold adaptation · Psychrophiles

Introduction

In eukaryotic cells, the α- and β-tubulins are highly conserved proteins. The two types of tubulins assemble into dimers, which in turn polymerize to form microtubules. These protein structures are known to play a major role in fundamental processes such as mitosis, meiosis, secretion, and cell motility. The assembly of microtubules is partly mediated by microtubule-associated proteins (MAPs). Detrich et al. (1989) have observed that brain tubulins from an Antarctic fish are able to polymerize at temperatures close to 0°C whereas the assembly of microtubules from purified mammalian brain tubulins occurs only at much higher temperatures. This capacity of psychrophilic tubulins to assemble at low temperature is mainly ascribable to the structure of tubulins themselves and not to the MAPs. It has been shown indeed that the activity of MAPs from Antarctic fishes is not greater at low temperature than that of mammalian MAPs. According to these authors, the polymerization of microtubules at low temperature most likely concerns protein domains that are involved in the interactions between tubulin dimers. Such cold adaptation might originate from changes in the primary structure or from posttranslational modifications of the α- or β-tubulins (Detrich 1997).

Very little work has been devoted to the molecular adaptation to low temperatures of proteins from psychrophilic photosynthetic organisms (Loppes et al. 1996; Devos et al. 1998). As an attempt to better understand the mechanism of cold adaptation at the molecular level, we have isolated and sequenced the α-tubulin genes from two psychrophilic algal species belonging to the genus *Chloromonas*. We then compared their deduced amino acid sequences to those of the corresponding proteins in mesophilic algae with the aim of determining substitutions that could account for cold adaptation.

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Materials and methods

Strains and culture conditions

Two isolates (named ANT1 and ANT3) belonging to the genus *Chloromonas* were collected on Petrel Island in the Antarctic region (60°40′ S, 40°01′ E), purified in the labora-

tory, and maintained on agar medium under axenic conditions. The taxonomic position of these flagellated unicellular algae is very close to that of algae belonging to the genus *Chlamydomonas*, from which they notably differ at the structural level by the lack of a pyrenoid. The ANT1 and ANT3 *Chloromonas* strains display maximal growth at 8°C and 4°C, respectively, and both stop growing at 16°C. They can therefore be considered as true psychrophiles. They were grown at 5°C in the HSM (Harris 1989) minimal medium routinely used for *Chlamydomonas reinhardtii*, under continuous light and air bubbling. In these conditions, both ANT1 and ANT3 divide once about every 24 h in the exponential phase of growth.

DNA manipulations

Total DNA from ANT1 and ANT3 was prepared according to Rochaix (1978) with minor modifications (Devos et al. 1998). Genomic libraries were constructed in λFIXII™ (Stratagene, La Jolla, CA, USA) according to manufacturer recommendations. The libraries were screened (Sambrook et al. 1989) with the 584-bp *Pvu*II fragment from *C. reinhardtii* α-tubulin cDNA (Silflow et al. 1985) in 50% formamide at 42°C with a final wash in 0.1 SSC (15 mM NaCl, 1.5 mM tri-sodium citrat, pH7) at 55°C. The DNA fragments from positive plaques were subcloned into pBluescriptKS plasmid (Stratagene). Plasmids were transformed into *E. coli* (Inoue et al. 1990) and prepared by the alkaline lysis method (Sambrook et al. 1989), followed by a further purification step (Nucleobond; Macherey-Nagel, Düren, Germany) when required. DNA sequencing was carried out on both strands by the dideoxynucleotide chain termination method using T7 sequencing kit (Pharmacia Biotech, Uppsala, Sweden) with universal or specific internal oligonucleotides (Eurogentec, Liege, Belgium). Sequence analyses were performed using the GCG system. The sequences of the ANT1 and ANT3 α-tubulin genes are available in GenBank under accessions AS032876 and AS032877, respectively.

Results and discussion

Cloning the genes and analysis of DNA sequences

After screening of the genomic libraries with the heterologous α-tubulin probe from *C. reinhardtii*, two families of clones were recovered in the ANT3 library (showing *Hin*fI fragments of 1100 and 1300 bp, respectively) while only one family of clones was found in the ANT1 library (showing a unique 2400-bp *Hin*fI fragment). These data suggest that ANT1 has only one α-tubulin gene and that ANT3 harbors two α-tubulin genes. This is in agreement with the results of Southern blots on genomic DNA (data not shown) indicating the presence of two *Hin*fl fragments in ANT3 and only one fragment in ANT1.

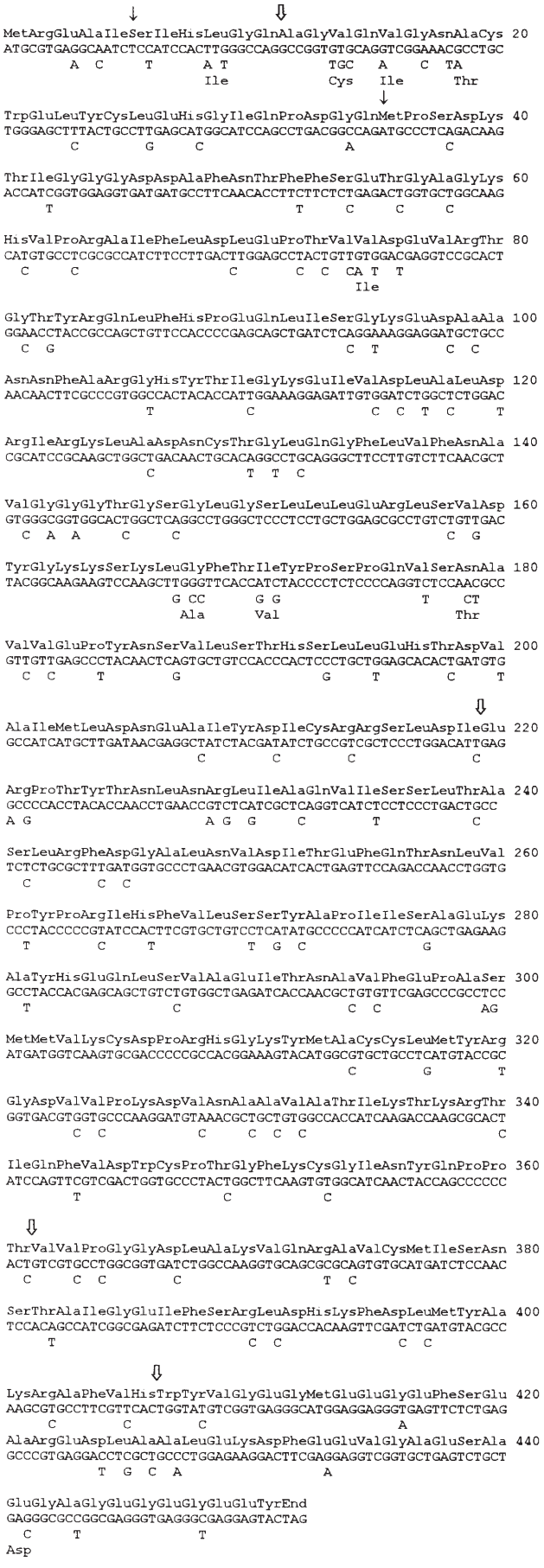
One clone derived from ANT1 and one clone derived from ANT3 were retained for further analysis. For each of them, several fragments covering the entire gene were subcloned into pBluescript plasmid and fully sequenced on both strands (Fig. 1). The positions of introns were inferred from the well-conserved consensus splice junctions (Mount 1982; Sharpe and Day 1993), and the amino acid sequences were deduced (Fig. 1). Two and four introns (ranging from 117 to 266 bp) were found in the ANT3 and ANT1 α-tubulin genes, respectively (data not shown). They are localized close to the 5′-end of the coding sequence in ant3 and are scattered throughout the whole sequence in ant1. No significantly conserved sequence was found in ant1 and ant3 introns. The coding regions of ANT1 and ANT3 α-tubulin genes each contain 1353 bp (predicting a product of 451 amino acids) with a G + C content of 59% and 61%, respectively. As also observed in the ant3 *rbcS* gene (Devos et al. 1998) and in most *C. reinhardtii* nuclear genes (Silflow et al. 1985), the codon usage is biased, 72% and 78% of amino acids being encoded by triplets ending with G or C in ant1 and ant3, respectively.

Comparing ANT1 and ANT3 α-tubulin sequences with other corresponding amino acid sequences

ANT1 and ANT3 α-tubulin coding sequences are 90% identical, the majority of base changes being substitutions at the third position of codons without modification of amino acid residues. At the polypeptide level, ANT1 and ANT3 amino acid sequences are remarkably similar and only present nine differences at positions 9, 14, 16, 19, 75, 168, 171, 179, and 441 (Fig. 1). ANT1 and ANT3 α-tubulin amino acid sequences were compared to the corresponding sequences of 12 representative mesophilic organisms: human, rat, *Drosophila*, salmon, *Tetrahymena pyriformis, Chlorella vulgaris, Chlamydomonas reinhardtii, Volvox carteri, Arabidopsis thaliana, maize, Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe.* This analysis revealed varying degrees of isology: 70% strict identities were found with the *S. cerevisiae* sequence, more than 88% with α-tubulins from *Arabidopsis* and *maize*, and more than 97% with the sequence common to the two algal species *C. reinhardtii* and *V. carterii*. As outlined by Arpigny et al. (1993), the identification of amino acid residues possibly involved in cold adaptation of proteins is often impaired by insufficient isology with their mesophilic counterparts. In the present case, the high degree of isology observed with algal tubulins is thus of great interest to determine which alterations of amino acid sequences might contribute to enhancement of microtubule polymerization at low temperature.

Comparing ANT1 and ANT3 α-tubulin sequences to the corresponding sequences in the phylogenetically related species *Chlamydomonas reinhardtii* and *Volvox carteri*

From a number of analyzed psychrophilic enzymes (for recent reviews, see Feller et al. 1996; Feller and Gerday 1997; Gerday et al. 1997), it is obvious that specific changes occurring in the primary structure of cold-adapted



proteins tend to lower the cohesion of the molecular structure and thus to increase the flexibility of the protein, enabling a better accommodation of the substrate at low temperatures.

To correlate the different amino acid substitutions observed in the Antarctic algae α-tubulins with cold adaptation, a description of the tridimensional structure of the αβ-tubulin dimer is required and is now available for pig brain tubulin (Nogales et al. 1998). The building block of microtubules is a heterodimer consisting of α- and β-tubulins (each about 50 kDa) displaying similar structures. Both α- and β-subunits may be subdivided into three major domains: a N-terminal, including residues 1–205, typical of nucleotide-binding proteins in which parallel β-strands alternate with α-helices; an intermediate domain formed by residues 206–381, made of a mixed β-sheet and five flanking helices; and a C-terminal domain formed by helices H11 and H12, overlaying the previous domains and identified as the outside surface of the microtubule.

The ANT1 and ANT3 α-tubulins were compared to the α_1-tubulin from *C. reinhardtii* and *V. carteri* (Fig. 2). Four substitutions are specific to ANT1 (at positions 9, 14, 171, and 179) and six to ANT3 (at positions 14, 16, 19, 75, 168, and 441). Five substitutions (at positions 4, 65, 202, 268, and 295) are common to both psychrophilic algae. The substitutions that are specific to either of the Antarctic algae command the following observations:

- Five substitutions ($I_9 \rightarrow L$, $I_{14} \rightarrow V$, and $V_{171} \rightarrow I$ in ANT1, $V_{16} \rightarrow I$ and $V_{75} \rightarrow I$ in ANT3) concern residues that are highly hydrophobic. The first substitution is probably neutral whereas positions 16 (helix 1), 75 (helix 2), and 171 (β-strand B5) are also occupied by I in the α-subunit of pig brain tubulin (Nogales et al. 1998). Therefore, the substitutions $V \rightarrow I$ are probably not involved in the adaptation to cold of Antarctic algae tubulins.
- One substitution ($T_{179} \rightarrow N$) in ANT1 occurs in a loop between B5 and H5 and concerns a strictly conserved position with regard to the α-tubulins from pig brain and from the 12 representative mesophilic organisms considered in this comparison (Nogales et al. 1998). This residue is located in the intradimer region, very close to the ribose of the bound GTP. The possible significance of this substitution is, however, unclear.
- The presence of a C at position 14 (helix 1) in ANT3 does not appear crucial because the same residue is found in *S. cerevisiae* α-tubulin.
- In ANT3, substitutions $A_{19} \rightarrow T$ in H1, $G_{168} \rightarrow A$ in B5, and $E_{441} \rightarrow D$ near the end of the C-terminal domain are also probably neutral.

The five substitutions common to ANT1 and ANT3 are possibly of greater interest as far as the adaptation to cold is concerned:

Fig. 1. Nucleotide and amino acid sequences from ANT1 (*upper two lines*) and ANT3 α-tubulin coding regions. Substitutions in ANT3 sequences are indicated in the *third* and *fourth lines*. The locations of introns are shown by *arrows*. ANT1, *open symbols*; ANT3, *filled symbols*

Fig. 2. Amino acid alignments of α-tubulins from the two psychrophilic strains ANT1 and ANT3, and from the mesophilic algae *Chlamydomonas reinhardtii and Volvox carteri*, whose sequences are identical. Variable residues are *shaded*

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                                                                                50
          ant1  MREAISIHLG QAGVQVGNAC WELYCLEHGI QPDGQMPSDK TIGGGDDAFN
          ant3  MREAISIHIG QAGCQIGNTC WELYCLEHGI QPDGQMPSDK TIGGGDDAFN
C.reinhardtii  MREVISIHIG QAGIQVGNAC WELYCLEHGI QPDGQMPSDK TIGGGDDAFN
  & V.carteri

                                                                               100
          ant1  TFFSETGAGK HVPRAIFLDL EPTVVDEVRT GTYRQLFHPE QLISGKEDAA
          ant3  TFFSETGAGK HVPRAIFLDL EPTVIDEVRT GTYRQLFHPE QLISGKEDAA
C.reinhardtii  TFFSETGAGK HVPRCIFLDL EPTVVDEVRT GTYRQLFHPE QLISGKEDAA
  & V.carteri

                                                                               150
          ant1  NNFARGHYTI GKEIVDLALD RIRKLADNCT GLQGFLVFNA VGGGTGSGLG
          ant3  NNFARGHYTI GKEIVDLALD RIRKLADNCT GLQGFLVFNA VGGGTGSGLG
C.reinhardtii  NNFARGHYTI GKEIVDLALD RIRKLADNCT GLQGFLVFNA VGGGTGSGLG
  & V.carteri

                                                                               200
          ant1  SLLLERLSVD YGKKSKLGFT IYPSPQVSNA VVEPYNSVLS THSLLEHTDV
          ant3  SLLLERLSVD YGKKSKLAFT VYPSPQVSTA VVEPYNSVLS THSLLEHTDV
C.reinhardtii  SLLLERLSVD YGKKSKLGFT VYPSPQVSTA VVEPYNSVLS THSLLEHTDV
  & V.carteri

                                                                               250
          ant1  AIMLDNEAIY DICRRSLDIE RPTYTNLNRL IAQVISSLTA SLRFDGALNV
          ant3  AIMLDNEAIY DICRRSLDIE RPTYTNLNRL IAQVISSLTA SLRFDGALNV
C.reinhardtii  AVMLDNEAIY DICRRSLDIE RPTYTNLNRL IAQVISSLTA SLRFDGALNV
  & V.carteri

                                                                               300
          ant1  DITEFQTNLV PYPRIHFVLS SYAPIISAEK AYHEQLSVAE ITNAVFEPAS
          ant3  DITEFQTNLV PYPRIHFVLS SYAPIISAEK AYHEQLSVAE ITNAVFEPAS
C.reinhardtii  DITEFQTNLV PYPRIHFMLS SYAPIISAEK AYHEQLSVAE ITNAAFEPAS
  & V.carteri

                                                                               350
          ant1  MMVKCDPRHG KYMACCLMYR GDVVPKDVNA AVATIKTKRT IQFVDWCPTG
          ant3  MMVKCDPRHG KYMACCLMYR GDVVPKDVNA AVATIKTKRT IQFVDWCPTG
C.reinhardtii  MMVKCDPRHG KYMACCLMYR GDVVPKDVNA SVATIKTKRT IQFVDWCPTG
  & V.carteri

                                                                               400
          ant1  FKCGINYQPP TVVPGGDLAK VQRAVCMISN STAIGEIFSR LDHKFDLMYA
          ant3  FKCGINYQPP TVVPGGDLAK VQRAVCMISN STAIGEIFSR LDHKFDLMYA
C.reinhardtii  FKCGINYQPP TVVPGGDLAK VQRAVCMISN STAIGEIFSR LDHKFDLMYA
  & V.carteri

                                                                               450
          ant1  KRAFVHWYVG EGMEEGEFSE AREDLAALEK DFEEVGAESA EGAGEGEGEE
          ant3  KRAFVHWYVG EGMEEGEFSE AREDLAALEK DFEEVGAESA DGAGEGEGEE
C.reinhardtii  KRAFVHWYVG EGMEEGEFSE AREDLAALEK DFEEVGAESA EGAGEGEGEE
  & V.carteri

                451
          ant1  Y
          ant3  Y
C.reinhardtii  Y
  & V.carteri
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- The substitution $V_4 \rightarrow A$ in B1 can potentially have a negative effect on the hydrophobic clusters maintaining the cohesion of the interaction between the internal β-sheet and the flanking helices. However, a cysteine occurs at this position in the pig brain α-tubulin so that the significance of this change is uncertain.
- Position 65 in β-strand B2 is occupied by an A in the Antarctic algae α-tubulins as well as in the pig brain α-tubulin. We therefore believe that the replacement $C_{65} \rightarrow A$ observed in psychrophilic algae has little to do with cold adaptation.
- Position 202 is in B6 leading to the intermediate domain. The replacement $V \rightarrow I$ has been found to be an important substitution in helical regions when one goes from thermophilic to mesophilic proteins (Argos 1989). It is a F in pig brain α-tubulin, and this position is very close to the intradimer contact region and GTP-binding sites. The substitution does not seem however to affect the interdimer interaction, being too close to the region of the intradomain contact to be involved in interdimer interactions.
- Position 268 is located in β-strand B7. The substitution $M \rightarrow V$ has never been encountered in the 12 representatives of mesophilic organisms. It corresponds in fact to an increase of the hydrophobicity and, surprisingly, a P is found at this position in pig brain α-tubulin. In the microtubule, this position is near the internal contact zone (241–256) between dimers (Arévalo et al. 1990). Moreover, other authors (Detrich and Parker 1993) have observed in the β-tubulin of an Antarctic fish a $M \rightarrow I$ substitution at position 267 equivalent to position 268 in α-tubulin. As the polymerization of tubulin from Antarctic fish has been found to be under entropic control as the result of an increase in number or in qualitatively stronger hydrophobic interactions at the level of the interdimer contacts (Detrich 1997), we believe that the substitution $M_{268} \rightarrow V$ can be part of the strategy leading to cold adaptation of microtubules from Antarctic algae.
- Finally, position 295 is the last residue of H9. It is also located in the interdimer contact region. The substitution $A \rightarrow V$ can give rise to a stronger hydrophobic interaction with respect to the adjacent protofilament as it has been postulated (Detrich et al. 1989) that, as in the case of the substitution $M_{268} \rightarrow V$, an increase in the hydrophobicity at contact regions may play a role in the cold stability of microtubules. This could appear at first sight paradoxical in view of the negative effect of low temperatures on hydrophobic interactions. It has been shown however (Detrich 1997) that the entropic control of microtubule polymerization increases with decreasing body temperature. At low and moderate temperature, hydrophobic interactions occur with a positive modification of enthalpy $\Delta H > 0$ and a largely positive modification of entropy $\Delta S > 0$ originating from the release of water forming initially a cage around exposed hydrophobic residues. The gain in free energy ΔG obtained in this way is largely independent of the environmental conditions contrary to what one would observe if hydrogen or electrostatic bonds were involved.

Thus, with the exception of substitutions at positions 268 and 295, which could play a significant role in the adaptation to low temperatures, the other changes common to ANT1 and ANT3 are largely internal and probably do not much alter the stability of the core. We cannot rule out however the importance of these changes in the modulation of the relative flexibility of the subunit as a whole.

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