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Cold adaptation of a psychrophilic chitinase: a mutagenesis study

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The gene encoding chitinase *Ar*ChiB from the Antarctic *Arthrobacter* sp. TAD20 has been expressed in *Escherichia coli* and the recombinant enzyme purified to homogeneity. In an effort to engineer cold-adapted biocatalysts through rational redesign to operate at elevated temperatures, we performed several mutations aiming to increase the rigidity of the molecular edifice of the selected psychrophilic chitinase. The mutations were designed on the basis of a homology-based three-dimensional model of the enzyme, and included an attempt to introduce a salt bridge (mutant N198K) and replacements of selected Gly residues by either Pro (mutants G93P, G254P) or Gln (G406Q). Mutant N198K resulted in a more stable protein (ΔT_m = 0.6°C). Mutant G93P exhibited a ΔT_m of 1.2°C, while mutants G254P and G406Q exhibited decreased stability. We conclude that the effect of mutating Gly residues on enzyme stability is rather complex and can only be understood in the context of the structural environment. Kinetic and spectroscopic analysis of these enzyme variants revealed that the kinetic parameters k_{cat} and K_m have been significantly modified.

Keywords: chitinases/cold adaptation/mutagenesis/psychrophilic enzyme/thermostability

Introduction

Cold-adapted enzymes, i.e. enzymes from organisms that constantly live at low temperatures, have evolved towards high catalytic efficiency in order to maintain adequate metabolic fluxes at the temperatures of their environment (Gerday *et al*., 2000). Numerous studies have shown that psychrophilic enzymes are generally characterized by (i) high turnover rates (k_{cat}) and catalytic efficiencies (k_{cat}/K_m) at low temperatures (<10°C) (Gerday *et al*., 2000), and (ii) reduced stability at moderate and high temperatures.

In many cases, adaptation of psychrophilic enzymes is believed to be achieved through a reduction in the activation energy (Miyazaki *et al*., 2000; Arnold *et al*., 2001), resulting from an increased structural flexibility of either selected residues, mainly located at the active site (Lonhienne *et al*., 2001a,b) or of the overall protein structure (Aghajari *et al*., 1998; Fields and Somero, 1998; Russell *et al*., 1998; Kim *et al*., 1999).

The reduced heat stability of psychrophilic proteins has been correlated with subtle changes of their sequences compared with mesophilic enzymes, such as decreased levels of Pro and Arg residues, increased numbers or clustering of Gly residues, weakening of intramolecular interactions, increased solvent interactions, decreased number of charged residue interactions and of disulfide bonds (Feller and Gerday, 1997; Feller *et al*., 1997; Marshall, 1997; Gerday *et al*., 2000; Russell, 2000; Lonhienne *et al*., 2001b).

It has recently been established that the two main characteristics of cold-adapted enzymes, i.e. high enzymatic activity and reduced structural stability, are not always linked (Miyazaki *et al*., 2000; Wintrode *et al*., 2001) and that their decreased stability may result from a random accumulation of destabilizing mutations, which are not incompatible with their enzymatic activity requirements at low temperatures (Feller and Gerday, 1997; Van den Burg *et al*., 1998). Furthermore, there is an increasing body of evidence that the reduced structural stability of psychrophilic enzymes and an increased structural flexibility are not necessarily related (Lonhienne *et al*, 2000; 2001b), although a close connection has been suggested in the past (Somero, 1975; Feller *et al*., 1996; Feller and Gerday, 1997).

In the present paper, we report a site-directed mutagenesis study of a cold-adapted chitinase, namely *Ar*ChiB. This study was performed with the double aim of testing theories relating stability and activity of psychrophilic enzymes and of providing new insights, which may form a basis for the re-engineering of their stability and activity parameters. Five *Ar*ChiB mutants were designed on the basis of first principles of protein structures and physicochemical properties of amino acids using sequence alignment and molecular modeling. The mutants were subsequently produced in *Escherichia coli* and their thermal stability and kinetic parameters were determined.

Chitinases are glycosyl hydrolases which catalyze the degradation of chitin, a fibrous, insoluble polysaccharide, consisting of β-1,4-*N*-acetylglucosamine residues. Chitin is the second most abundant polysaccharide on earth and chitinases are thought to be important in the digestion of chitin for utilization as a carbon and energy source (Yu *et al*., 1993).

Cloning and sequencing of the *archi*B gene from the Antarctic bacterium *Arthrobacter* sp. str. TAD20 have been previously reported (Lonhienne *et al*., 2001a). The enzyme belongs to glycosyl-hydrolases family 18 (Henrissat and Bairoch, 1996) and consists of a catalytic domain and one chitin binding domain. It exhibits the typical characteristics of cold-adapted enzymes, i.e. higher catalytic efficiency and decreased stability, compared with mesophilic chitinases (Feller and Gerday, 1997; Gerday *et al*., 2000).

Materials and methods

All DNA manipulations were performed using standard methods (Sambrook *et al*., 1989). Restriction and DNA modification enzymes, and Vent polymerase were purchased from New England Biolabs (Beverly, MA) and MINOTECH (Heraklion, Greece). All chemicals were of analytical grade for biochemical use. PCR primers were purchased from the Microchemistry Laboratory of IMBB. Sequencing reactions were performed in a PE-AB1377 DNA sequencer in the Laboratory of Applied Entomology (IMBB).

Enzyme assay

Chitinolytic activity was routinely assayed at 25°C using 0.1 mM pNP-chitotriose (*p*-nitrophenyl *N*,*N*′,*N*"-triacetyl-chitotriose) (Sigma) as substrate for *Ar*ChiB, in 50 mM HEPES, pH 7.5. Activities were recorded in a thermostated Lamda 2 spectrophotometer (Perkin Elmer) and calculated on the basis of an extinction coefficient for *p*-nitrophenol of 14 700 M^{-1} cm^{-1} at 405 nm.

Cloning of archiB gene

The *archi*B gene was cloned in pET26b vector downstream of the pelB leader peptide. The gene was amplified using PCR from the library clone using the primers ChiBforward and ChiBreverse.

Expression and purification of the recombinant enzymes

Expression of the native *Ar*ChiB enzyme and enzyme variants was performed in Bl21(DE3) *E.coli* cells. Four liters of culture were grown at 20°C and induced when OD_{600} was 0.6–0.8 using 0.1 mM IPTG. The culture was centrifuged after 12 h and the supernatant containing *Ar*ChiB was concentrated to a final volume of 200 ml. Proteins were precipitated using 1.8 M $(NH_4)_2SO_4$ and the sample was centrifuged for 1 h at 10 000 r.p.m. in a JA-10 rotor on a Beckman Model 32-21 centrifuge. The pellet was resuspended and $(NH_4)_2SO_4$ was added to a final concentration of 1 M. The sample was centrifuged and the supernatant was loaded on a phenyl-Sepharose fast-flow column, previously equilibrated with a buffer containing 1 M $(NH_4)_2SO_4$. An $(NH_4)_2SO_4$ gradient (1–0 M) was applied. The column was eluted with a buffer containing 50 mM HEPES, pH 7.4. Fractions exhibiting chitinase activity were pooled and concentrated to a final protein concentration of 1.5 mg/ml.

Thermal inactivation

In order to measure the thermal inactivation of enzymes, 1.5 μg of the purified enzymes (0.1 μg/μl) were incubated at 45°C in a 50 mM HEPES buffer for different time periods (30 and 60 min) and they were subsequently incubated on ice for 30 min. The residual activity was measured at 20°C. The reported values are the average of five measurements. The standard deviations did not exceed 10%.

Fluorescence

Heat-induced unfolding was recorded by monitoring the fluorescence of the samples using an SML-AMINCO Model 8100 spectrofluorimeter (Spectronic Instruments) at an excitation wavelength of 280 nm and an emission wavelength of 340 nm. In order to reduce photobleaching of the samples, the excitation band-pass was kept at 1 nm (4 nm for emission) and was closed between records. Samples (1 ml) in 50 mM HEPES, pH 7.5 were adjusted to A_{280} = 0.1 and were heated in the fluorimeter cell at a rate of 2°C/min using a programmed water bath (Lauda Ecoline RE306). The temperature of the sample was recorded in the cell by a copper–constantan thermocouple connected to a calibrated digital thermometer (Thermo Electric). Data were normalized using the pre- and post-transition baseline slopes as previously described (Pace, 1986).

Site-directed mutagenesis

Mutants were constructed by a two step–four primer overlap/extension PCR method (Horton *et al*., 1990). The amplified product was subcloned in pET26b vector downstream of the pelB signal peptide. The primers used for the mutations were as follows: ChiBforward, CTGCACCGCCCAACACCGC; ChiB-reverse, GCAGGAATTCGATCTAGTT; G93Pforward, TTC-GGCATGCCTTATGCGGCCGACAAGTCC; G93Preverse, GGCCGCATAAGGCATGCCGAAGTCTGCCCA; N198K-forward, CGGGCACAAAGTCCGGTCTAGAAGGCAACG; N198Kreverse, TTCTAGACCGGACTTTGTGCCCGGCCA-TTC; G254Pforward, CGATGCAGGTCCCTGGGATGA-CCCTGCCAACT; G254Preverse, GTCATCCCAGGG-ACCTGCATCGATGTCCGCAG; G406Qforward, TGTCAG-CAAGCAGCTTGGCGGCGGCATGTGGT; G406Qreverse, CGCCGCCAAGCTGCTTGCTGACAATGTAGTCA; Cys57-forward, CGGTAACATCTGCAACAACCAGACATTGAC-CTG; Cys57reverse, CTTGGTTGTTGCAGATGTTACCGA-AAAGAGTAGT; Cys80forward, CCCAACGGCTCCTGT-GATGGCGCCGGCGATGCT; Cys80reverse, GGCGCCAT-CACAGGAGCCGTTGGGACCGGTTCC.

Homology modeling

The three-dimensional (3D) structural model of the catalytic domain of *Ar*ChiB was built on the basis of the homology to the catalytic domain of *Sm*ChiA (overall similarity 33%) (Perrakis *et al*., 1994). Sections of the *Ar*ChiB catalytic domain sequence, which showed similarity of more than 50% to the *Sm*ChiA catalytic domain, were subjected to SwissModel (Guex and Peitsch, 1997). Missing loops in the new model were generated by sequence-based comparison with known loop structures, and based on the position of the α-carbon atoms in the loops. Regions lacking side chains were modeled using a library of allowed chain rotamers. The ultimate model was subjected to energy minimization using XPLOR (Brunger, 1992). For display of the model and for design and analysis of the estimated effect of the mutations the program SwissPDB (Guex and Peitsch, 1997) was used.

Steady-state enzyme kinetics

Steady-state enzyme kinetics were performed in the temperature range 12–30°C. The program Hyper.exe (v. 1.01) was used for the determination of v_{max} and K_m values. The k_{cat} values were calculated from v_{max} using a molecular weight for the enzyme of 56 640 Da. The reported values are the average of three measurements. The standard deviations did not exceed 5%. The thermodynamic parameters were calculated according to Lonhienne *et al.* (Lonhienne *et al*., 2000)

N-terminal amino acid analysis

The protein was sequenced using an ABI 476A automated protein sequencer (Applied Biosystems, Foster City, CA) with on-line detection of the PTH amino acids.

Mass spectrometry

Mass spectrometric analyses were performed on a Q-TOF hybrid quadrupole-time-of-flight instrument (Micromass, Manchester, UK) equipped with a nano-electrospray source.

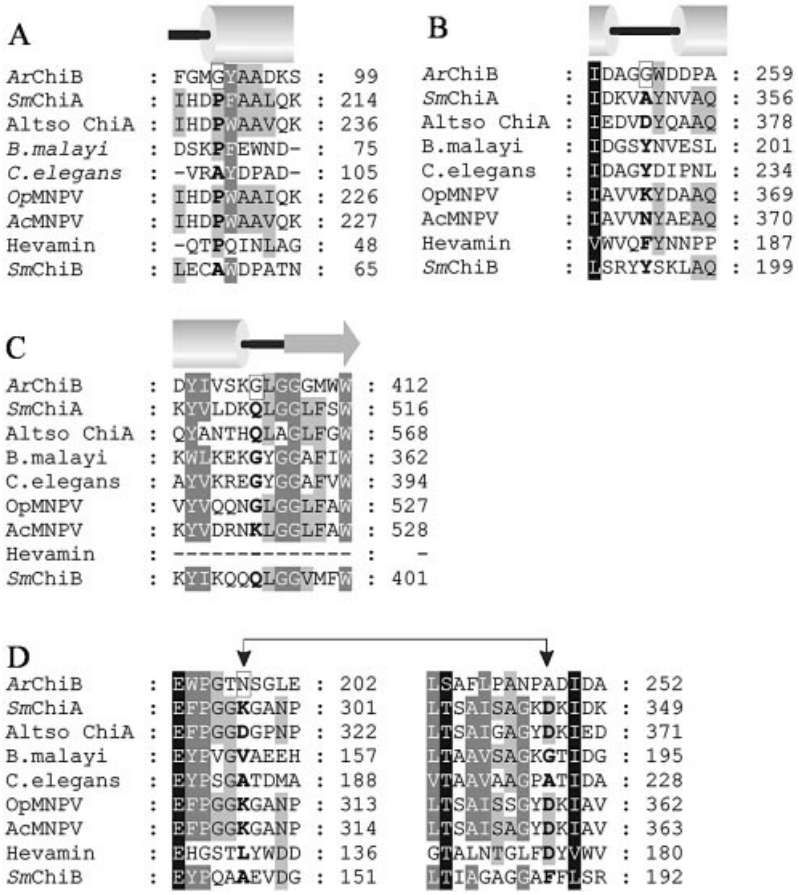

Fig. 1. Partial alignment of *Ar*ChiB with other chitinases. The secondary structure of *Sm*ChiA is indicated above the sequences (cylinder, α-helix; arrow, β-sheet; thick line, loop region). Mutation targets are in boxes. (**A**) Gly93 is mutated to Pro; (**B**) Gly254 is mutated to Pro; (**C**) Gly406 is mutated to Gln. (**D**) The double arrow indicates the interaction between Lys and Asp that forms a salt bridge in the structure of *Sm*ChiA. Asn198 is substituted by Lys in an attempt to restore the salt bridge in the *Ar*ChiB structure.

Typically 5 µl of a 1 pmol/µl protein solution in acetonitrile/0.1% formic acid in water (1:1) was loaded in a borosilicate capillary coated with Au/Pd (Protana, Odense, Denmark) to which 1.1 kV was applied. Spectra were accumulated during 3 min. The instrument was calibrated using horse myoglobin prior to measurement

Results

Purification and characterization of the enzymes

Following induction with IPTG the cloned *archi*B gene and each mutant were expressed in *E.coli* and the recombinant native and modified forms of the enzyme were secreted to the liquid culture media. Using the scheme described in Materials and methods, recombinant *Ar*ChiB and all the mutants were purified to homogeneity, with a total yield of ~15 mg/l.

N-terminal amino acid analysis of the purified *Ar*ChiB protein revealed the following amino acid sequence: H_2N–AlaAspGlyProLeu. Purified *Ar*ChiB on 12% SDS gel exhibited an apparent molecular weight of ~80 kDa.

The calculated molecular weight from the gene translation of the mature protein is 56 442 Da. Mass spectrometry experiments indicate a molecular weight of 56 440 ± 5 Da for *Ar*ChiB.

The enzyme exhibits the highest activity at pH 7.3–8 in HEPES buffer. The p*I* of the protein is 7.8 as judged from IEF electrophoresis.

Choice of mutations

Multiple sequence alignment of the available chitinases was used to reveal residues that are potentially involved in cold adaptation and stability of the protein (Figure 1). Molecular modeling was used as an additional tool in the design of mutations. First principles concerning protein structures and stability were applied as a guide for specific amino acid substitutions, thus providing a rational approach towards understanding psychrophilicity.

The overall aim was to attempt to rigidify the molecular edifice and to test the implications on enzymatic activity. The first principle used is to replace Gly residues. These residues have more conformational freedom than any other amino acid (Branden and Tooze, 1999). A Gly residue at a specific position in a protein has usually only one conformation in the folded structure, but can have many different conformations in different unfolded structures of the same protein and thus contribute to the diversity of unfolded conformations, thereby increasing the conformational entropy of the unfolded state. All other residues, and most prominently Pro, have less conformational freedom in the unfolded state. The following Gly replacements were performed.

Gly93Pro. Position 93 is in a conserved region among chitinases and is predicted to be at the beginning of an α-helix as judged from *Sm*ChiA. Pro is known to be a good helix starter while Gly to Pro substitutions generally have stabilizing effects

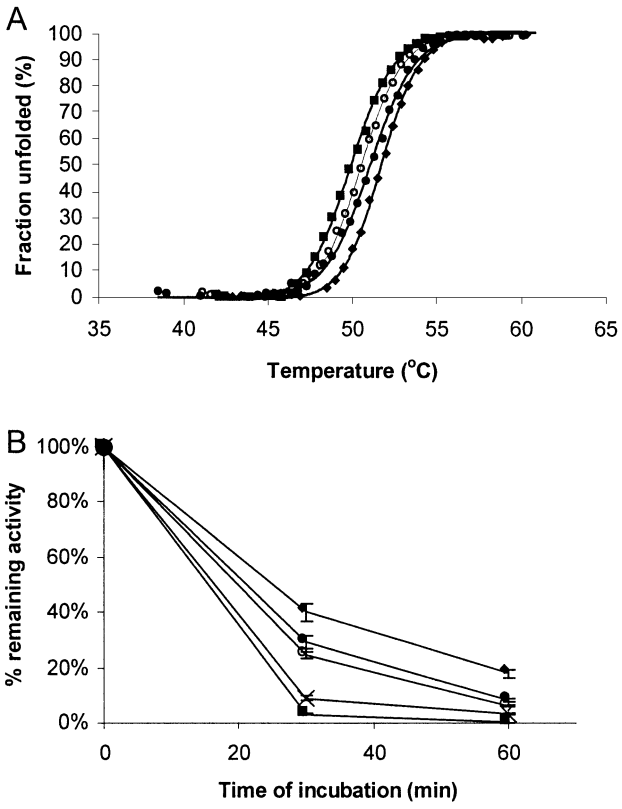

Fig. 2. (**A**) Thermal unfolding of *Ar*ChiB and variants recorded by fluorescence. The T_m values of *Ar*ChiB (open circles), Gly93Pro (diamonds), Asn198Lys (closed circles) and Gly406Gln (squares) are 50.5, 51.7, 51.1 and 49.9°C, respectively. (**B**) Residual activity of the proteins after incubation at 45°C for 30 and 60 min; symbols are as in (A); (crosses) Gly254Pro.

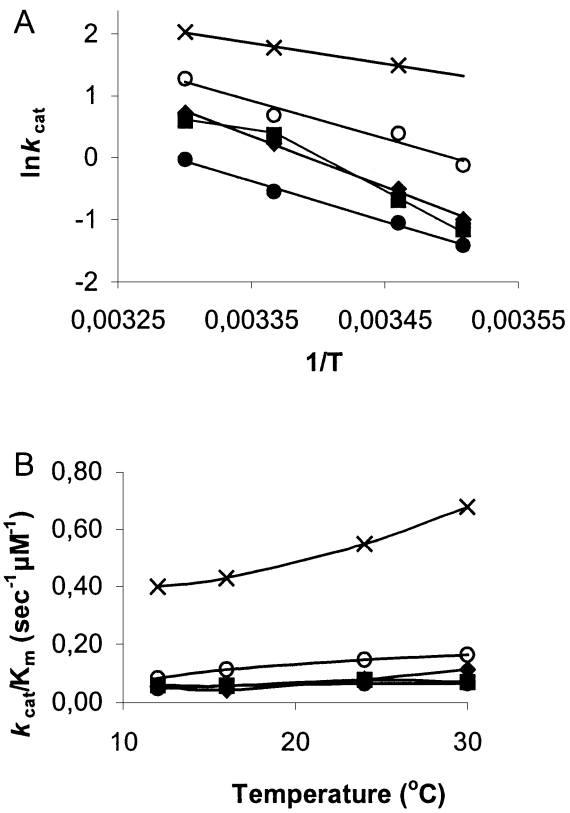

Fig. 3. (**A**) Arrhenius plots used to calculate the E_a for *Ar*ChiB and variants, for the temperature range 12–30°C. k_{cat}/K_m for *Ar*ChiB (open circles), Gly93Pro (diamonds), Asn198Lys (closed circles), Gly406Gln (squares) and Gly254Pro (×). (**B**) Temperature dependence of catalytic efficiency of *Ar*ChiB and variants; symbols are as in (A).

(Branden and Tooze, 1999). Furthermore, Pro residues at position 93 are found in several other chitinases (Figure 1A).

Gly254Pro. Position 254 is located in a loop region connecting two α-helices and is positioned next to another Gly belonging a Gly cluster. Sequence alignment with other chitinases revealed that this cluster is unique to the enzyme under study and that position 254 is often occupied by less flexible residues (Figure 1B). We substituted Gly at this position with Pro because of its rigidifying effects; furthermore, on the basis of the ϕ/ψ conformational angles, the local geometry at position 254 is compatible with Pro (although sequence alignments do not show Pro at an equivalent position in other chitinases).

Gly406Gln. Position 406 is in a well conserved region close to a Gly cluster and is often occupied by large residues such as Gln and Lys (Figure 1C). Interestingly, Gly at this position is frequently observed in other chitinases. Pro is not compatible with the local conformation in the homology-based model, thus Gly was substituted by Gln.

The second principle used in the design of mutations was based on a statistical analysis of protein sequences. In this context, it was attempted to introduce additional interactions, e.g. salt bridges, which occur statistically less often in psychrophilic enzymes compared with their mesophilic counterparts (Feller and Gerday, 1997; Gerday *et al*., 2000). Sequence alignments combined with the homology-based 3D model provided a basis to propose specific mutations which could give rise to salt bridge formation. The following mutations were performed.

Asn198Lys. In the case of the mesophilic chitinases positions 198 and 248 (using the numbering of the *Ar*ChiB sequence) are often occupied by Lys and Asp, respectively; in *Sm*ChiA these residues form a salt bridge (Figure 1D). Assuming that the 3D structure of the cold-adapted enzyme is sufficiently conserved in this region, we tried to introduce a salt bridge by mutagenesis. Since position 249 is occupied by Asp, we substituted Asn198 with Lys aiming to introduce an interaction between Asp249 and Lys198. This interaction was strongly suggested by the homology-based 3D model of the psychrophilic enzyme.

The third principle used in the design of mutations was to introduce a disulfide bridge and thereby decrease the conformational entropy of the unfolded state. Insertions of Cys following residues 56 and 79 were prepared based on alignment with the *Sm*ChiA. This mutant did not produce any active chitinase.

Fluorescence

The melting temperature of *Ar*ChiB was determined as 50.5°C. Mutants Asn198Lys and Gly93Pro exhibit a higher T_m, by 0.6 and 1.2°C, respectively. In contrast, mutant Gly406Gln exhibits a lower T_m, by 0.6°C (Figure 2A). Due to the extreme instability of mutant G254P it was not possible to determine the T_m value of this mutant, as it was rapidly deactivated.

Table I. Enzymatic and thermodynamic parameters of the psychrophilic chitinase and mutants

	E_a (kJ/mol)	$\Delta G^{\#}$ (kJ/mol)	$\Delta H^{\#}$ (kJ/mol)	$T\Delta S^{\#}$ (kJ/mol)	$\Delta(\Delta G^{\#})_{n-m}$ (kJ/mol)	$\Delta(\Delta H^{\#})_{n-m}$ (kJ/mol)	$T\Delta(\Delta S^{\#})_{n-m}$ (kJ/mol)
*Ar*ChiB	50.7	69.8	48.3	–21.5			
G93P	67.9	71.9	65.5	–6.4	–2.2	–17.2	–15.0
N198K	53.9	73.2	51.5	–21.7	–3.5	–3.2	0.3
G254P	27.8	67.1	25.4	–41.7	2.6	22.9	20.3
G406Q	73.5	72.4	71.1	–1.3	–2.6	–22.8	–20.2

The reported values were determined at 16°C. The k_{cat} values were calculated from v_{max} using a molecular weight for the enzyme of 56 640 Da in a buffer containing 50 mM HEPES, pH 7.5. E_a values were calculated from the slope of the Arrhenius plots in the temperature range 12–30°C. Thermodynamic parameters $\Delta G^{\#}$, $\Delta H^{\#}$ and $T\Delta S^{\#}$ were calculated according to Lonhienne *et al*. (Lonhienne *et al*., 2000).

Thermal inactivation

In order to measure the effect of each mutation on the stability of the enzyme the residual activity after incubation at 45°C for 30 and 60 min was measured. The results are shown in Figure 2B.

Mutant Gly93Pro shows significantly higher levels of residual activity as compared with the recombinant enzyme, mutant Asn198Lys slightly higher compared with the native enzyme, while all other mutants exhibit lower residual activity.

Determination of E_a and thermodynamic parameters for wild-type and mutant enzymes

In order to elucidate the effect of mutations in terms of psychrophilic adaptation, we determined the energy of activation E_a for wild-type and mutant enzymes. Figure 3A shows the Arrhenius plots for the temperature range 12–30°C. The E_a of the enzymes reveals that the mutants Gly406Gln and Gly93Pro exhibit higher values than the native cold-adapted enzyme (Table I). The mutant Gly254Pro exhibits an E_a almost half that of the native enzyme while Asn198Lys exhibits almost the same E_a as the native enzyme (Table I).

Kinetic parameters

The specific activity of all mutants was measured over the entire range of temperature (12–30°C) (Figure 3B). The mutant Gly406Gln exhibits an increase in k_{cat}/K_m, while all other mutants exhibit a reduced catalytic efficiency compared with *Ar*ChiB (Figure 3B). The calculated values for E_a for each protein are shown in Table I.

Discussion

The gene encoding chitinase *Ar*ChiB from the Antarctic *Arthrobacter* sp. TAD20 has been expressed in *E.coli* and the recombinant enzyme purified to homogeneity. More than 90% of enzyme activity was detected in the culture media, although the protein was cloned in pET26b vector downstream of the pelB leader peptide and was therefore expected to be secreted in the periplasm. Furthermore, the N-terminal amino acid analysis of the recombinant protein revealed that the protein is seven amino acid residues shorter than the native protein. This could be the result of a proteolytic cleavage resulting in secretion of the enzyme. However, secretion of recombinant proteins is not unusual, but in most cases the mechanism is not known (Makrides, 1996).

The observed molecular weight of *Ar*ChiB on SDS gels is higher than the molecular weight calculated by mass spectrometry. This has also been observed in the case of the native enzyme (Lonhienne *et al*., 2001a) and might be due to the electrostatic charge and conformation of the enzyme in SDS polyacrylamide gels.

Five *Ar*ChiB mutants were designed aiming to increase the rigidity of the molecular structure of the enzyme either by introducing a salt bridge or by replacing selected Gly residues by less flexible ones. Mutations were restricted to sites where local homology is high, and consequently the 3D model is more accurate. Mutation Gly254Pro results in the elimination of a Gly cluster. Gly clusters have been frequently described as essential features for cold adaptation (Feller and Gerday, 1997; Gerday *et al*., 1997).

The double mutant Cys57/Cys80, which was constructed in order to introduce a disulfide bridge by analogy to *Sm*ChiA, did not exhibit any chitinolytic activity, while the protein corresponding to this *Ar*ChiB mutant could not be detected (data not shown). Introduction of disulfide bridges is quite complex in protein engineering (Narinx *et al*., 1997), often leading to misfolded proteins. Since two Cys residues are present in *Ar*ChiB, this mutation probably results in a non-native arrangement of cysteines, leading to an inactive and possibly aggregated product.

All mutants compared with the cold-active recombinant enzyme exhibit alterations in T_m and in the thermal inactivation profile (Figure 2A and B). Two of the mutants, Gly93Pro and Asn198Lys, exhibit higher T_m, while mutants Gly406Gln and Gly254Pro exhibit lower T_m (Figure 2A).

Mutant G93P targets one residue at the start of an α-helix. This residue is very suitable to study one important biophysical property of psychrophilic enzymes, i.e. the effects of altering the local conformation freedom of the protein in the unfolded state. This mutant is more stable than the recombinant *Ar*ChiB, exhibiting an increased T_m value by 1.2°C. Measurement of the residual activity of this mutant, following incubation at 45°C, is consistent with the observed increase in T_m (Figure 2A). We conclude, therefore, that the introduction of Pro at this position induces, as expected, an increase in enzyme stability (Figure 2). Furthermore, the E_a of this enzyme variant is higher as compared with that of *Ar*ChiB (67.9 versus 50.7 kJ/mol, respectively). Both properties of this mutant, i.e. stability and enzymatic activity, are shifted towards more mesophilic values, therefore, suggesting that the psychrophilic character of the enzyme has been altered to a more mesophilic one (Table I). As shown in Table I, this is mainly attributed to the considerable increase of ΔH of the mutant compared with the native enzyme (Lonhienne *et al*., 2000). Although position 93 is distant from the active site, the catalytic efficiency of the mutant is halved at all temperatures tested (Figure 3B).

Mutant Asn198Lys exhibits higher stability than recombinant *Ar*ChiB (T_m increases by 0.6°C, Figure 2A). This is comparable to the stabilization effects due to the introduction of salt bridges (D'Amico *et al*., 2001). The modeled structure

suggests that this mutation probably establishes a salt bridge between Lys198 and Asp249, which is present in the structure of the mesophilic protein *Sm*ChiA (Figure 1D). In this mutant, the E_a value (53.9 kJ/mol, Table I) is close to that of the *Ar*ChiB protein, suggesting that the psychrophilic character is conserved. Also in this case, the catalytic efficiency is lowered almost 2.5 times (Figure 3B). Position 198 is in the proximity of the active site and this mutation could both affect the flexibility and charge of the active site.

In mutant G254P, two effects, which are considered to affect psychrophilicity negatively, are simultaneously explored, i.e. the reduction of the local conformation freedom of the protein in the unfolded state (due to P) is combined with the elimination of one Gly cluster (a frequent feature of the sequences of psychrophilic enzymes). Gly254Pro exhibits a lower stability compared with *Ar*ChiB, as judged from the thermal inactivation profile (Figure 2B). This mutant clearly demonstrates that elimination of a glycine cluster and reduction of the local conformational freedom is not necessarily accompanied by an increased stability. E_a is lower than that of the *Ar*ChiB (Table I), suggesting that the psychrophilic character has been preserved and even more enhanced. This is mainly attributed to the considerable decrease of ΔH of the mutant compared with the native enzyme and a concurrent increase of ΔS (Lonhienne *et al*., 2000). The mutant also exhibits a 4-fold increase of catalytic efficiency (Figure 3B). Thus, for mutant Gly254Pro, stability and catalytic activity are both shifted towards more psychrophilic values through the elimination of the Gly cluster. The reason for this behavior is poorly understood; we suspect that local topology (position 254 is in a loop region) could be part of the answer, but this will have to be explored further. In contrast, recent studies of the psychrotolerant hormone-sensitive lipase and cold-adapted alkaline phosphatase have demonstrated that the presence of Gly clusters affects the activity rather than the stability and psychrophilic character (Laurell *et al*., 2000; Mavromatis *et al*., 2002) of the enzyme.

Mutant Gly406Gln affects a well conserved region of the protein sequence. This enzyme reveals a dichotomy between activity and stability behavior. The mutant has a reduced stability compared with the recombinant *Ar*ChiB enzyme (Figure 2A), while interestingly the E_a value has increased, reaching values typical for mesophilic enzymes (Table I). Furthermore, a bend in the Arrhenius plot is observed at elevated temperatures (Figure 3A) and this could be consistent with the establishment of stereochemical constraints due to this mutation (Tsigos *et al*., 2001; Mavromatis *et al*., 2002). The behavior of the Gly406Gln variant could thus partially reflect sterical clashes introduced by the Gln side chain. At higher temperatures the additional energy provided to this residue by the environment could be sufficient to enable the catalytic process to proceed as efficiently as in the wild-type protein. Overall, our observations suggest that Gly at position 406 plays a key role in the establishment of the psychrophilic character. The mutant Gly406Gln shows that for some residues it is possible to introduce mutations which alter stability and activity in a way that contradicts widely accepted definitions of mesophilic–psychrophilic enzyme properties.

The role of the Gly residues and Gly clusters is thus complex and substituting these residues with less flexible ones can produce enzymes with altered E_a values and thermal stability depending on their positions and their local environments, in agreement with previous observations (Laurell *et al*., 2000; Masumoto *et al*., 2000; Mavromatis *et al*., 2002). Introduction of a salt bridge can also produce a more stable enzyme.

The mutation targets (except Asn198) were located far from the active site as modeled in the present work. This suggests that primary structure changes that occur far from the active site display a complex effect on stability, activity and thermodynamic parameters. This behavior, which could reflect local changes in conformational flexibility, is in agreement with results of earlier studies (Grutter and Matthews, 1982; Bonomo *et al*., 1995; D'Amico *et al*., 2001).

In conclusion, we designed mutants aiming to rigidify the molecular edifice, and furthermore, to examine their role in the establishment of the psychrophilic character of the enzyme. Mutations of selected Gly residues, introduction of a salt bridge, as well as the formation of a disulfide bridge, were examined. Two of the mutants, namely Gly93Pro and Asn198Lys, exhibited increased stability as compared with the recombinant *Ar*ChiB. Furthermore, in all cases investigated, except for the mutant Gly406Gln, the proteins maintain their psychrophilic character. However, there is no straightforward relationship between flexibility, stability and activity since each mutation contributes differently to adaptation, sometimes in the opposite direction, suggesting that mutations accumulated during evolution act in a strong synergistic way.

In agreement with previous studies (Tsigos *et al*., 2001; Mavromatis *et al*., 2002), we conclude that a homology-based model of an enzyme (in the present work of the catalytic domain) is frequently sufficient for the rational design of mutations that affect heat stability and the psychrophilic character of the enzyme, if basic physicochemical properties of the amino acids are taken into account.

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