

Decreasing the stability and changing the substrate specificity of the *Bacillus stearothermophilus* alcohol dehydrogenase by single amino acid replacements

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The gene encoding the alcohol dehydrogenase (*adh-hT*) from the thermophilic bacterium *Bacillus stearothermophilus* LLD-R strain has been overexpressed in *Escherichia coli* and the corresponding recombinant protein purified to homogeneity. Two putative structural determinants contributing to the higher stability of ADH-hT had been identified by comparison with the less thermostable ADH (ADH-T) from the less thermophilic *B.stearothermophilus* NCA 1503. In order to ascertain their role, mutations were designed to eliminate in ADH-hT a salt bridge at the N-terminus and a proline residue in the coenzyme binding domain replacing the amino acids located at the same positions in ADH-T. Three mutants—Glu11Lys, Pro242Ala, and Glu11Lys/Pro242Ala—were expressed at high level and the proteins purified and characterized. In general, the mutations had little effect on the activity, indicating that they were not disruptive. The thermal resistance was changed displaying quite additive effects.

Keywords: alcohol dehydrogenase/expression/site-directed mutagenesis/thermal adaptation

Introduction

Studies on protein stability have been almost exclusively carried out by either studying the thermodynamics of protein unfolding (Privalov and Makhatadze, 1993) or by evaluating the functional effects of single or multiple selected amino acid substitutions in the primary structure of a protein by site-directed mutagenesis (Fersht and Serrano, 1993). Statistical and computational approaches were also recently demonstrated to be very effective in the prediction of mutant protein stability (Menéndez-Arias and Argos, 1989; Topham *et al.*, 1997). Moreover, the comparative analysis of proteins displaying similar functions in organisms growing at different temperatures represents a valid alternative to determine the molecular mechanisms responsible for the adaptation of proteins to different conditions (Vieille and Zeikus, 1996).

An alcohol dehydrogenase system was chosen to investigate the molecular determinants of protein thermostability. Alcohol dehydrogenases (ADH E.C. 1.1.1.1.) catalyse the reversible oxidation of alcohols to the corresponding aldehydes or ketones, with coenzyme and substrate specificity depending on the specific ADH (Brändén *et al.*, 1975). The medium chain zinc-containing ADHs, when compared with the long and short chain families (Jörnvall *et al.*, 1987) have been studied in greater detail, since either they are widely distributed

among all living organisms or play an essential role as universal factors in cellular defence mechanisms from prokaryotes to humans (Danielsson *et al.*, 1994). The considerable interest in their molecular biology, especially for those isolated from thermophilic sources, can be attributed both to their potential for biotechnological applications (Coolbear *et al.*, 1992; Cowan, 1992; Adams *et al.*, 1995) and the capability of elucidating their mechanism of action, regulation and evolutionary relationships.

Recently, Cannio and co-workers (1994) analysed the relationship between thermostability and the secondary structures predicted from the sequences of two different thermophilic ADHs: ADH-hT from *Bacillus stearothermophilus* LLD-R (optimum growing temperature 75°C) and ADH-T (Sakoda and Imanaka, 1992; Guagliardi *et al.*, 1996) from the less thermophilic strain NCA 1503 of *B.stearothermophilus* (optimum growth temperature 55°C), and suggested that the increase in thermostability of ADH-hT could be due, among the 31 substitutions, to a few amino acids occurring at significant positions in the sequence (Cannio *et al.*, 1994).

The substitutions occurred in two different putative structural determinants of the protein. The structural motifs for the protein under study were extrapolated from the X-ray structure of the horse liver ADH (Eklund *et al.*, 1976); in fact although some thermophilic ADHs have been recently crystallized (Pearl *et al.*, 1993; Zhang *et al.*, 1993; Korkhin *et al.*, 1996), their overall 3D structure is not currently available. The comparison based on the alignment of the sequences revealed as particularly interesting the presence in ADH-hT of two residues, Glu11 and Lys14, positioned in the N-terminus α -helix and replaced by a lysine and a glutamine located at the same position in ADH-T.

Recent high resolution X-ray studies on a number of thermostable proteins pointed out the contribution of charged side-chains to thermal stability by the generation of an ionic network (Ishikawa *et al.*, 1993). The role of ionic networks in the thermal stability of proteins has also been recently functionally demonstrated by site-directed mutagenesis through which specific salt bridges in α -helices have been disrupted (de Prat Gay *et al.*, 1994; Pappenberger *et al.*, 1997).

The residue Glu11, present in the more thermostable ADH-hT, is very close to the positive N-terminus pole of the helix, so that it can stabilize the helix by balancing the dipole effect (Nicholson *et al.*, 1988). Moreover, the same residue generates an additional surface salt bridge with the Lys14 enhancing the intrachain interaction in the helix (Marqusee and Baldwin, 1987).

An additional interesting substitution was observed in position 242 where a proline in ADH-hT was replaced by an alanine located in ADH-T in the same position. The proline 242 was found in ADH-hT in a flexible loop connecting a β -strand to a α -helix in the coenzyme binding domain. Proline is known to impose rigidity to polypeptide chains especially when it substitutes small amino acids in loops between elements

of secondary structures (Watanabe *et al.*, 1994). Moreover, it has been demonstrated that protein thermostability can be increased by mutations that decrease the configurational entropy of unfolding (Matthews *et al.*, 1987) and decreased by site-directed mutations that remove proline residues at specific positions (Herning *et al.*, 1992). Therefore, proline 242 can stabilize ADH-hT with respect to ADH-T by both conferring rigidity to the entire polypeptide chain and by stabilizing it through the reduction of the entropy of the unfolded state.

In order to verify these hypotheses, sufficient material for study was obtained by the high level expression of the *adh-hT* gene in *E.coli*. The amino acid residues (Glu11, Pro242) presumed to contribute to the higher thermostability of ADH-hT were substituted by the amino acids present at the same position (respectively Lys11, Ala242) in the less thermostable ADH-T by site-directed mutagenesis, and the resulting mutated genes expressed in *E.coli*. All the recombinant proteins were expressed, purified, and their stability measured as a function of temperature.

The point mutations were found to decrease the thermal stability but not the thermal activity of the mutants. We also demonstrated that the mutations introduced could influence the substrate specificity and the catalytic efficiency in different manners.

Materials and methods

Construction of the expression vector

Isolation of the *adh-hT* coding sequence was obtained by PCR; the template was the *adh-hT* gene previously isolated from a *B.stearothermophilus* gene library constructed in *E.coli* (Cannio *et al.*, 1994). The 5' primer used in the amplification (GenSet) contained the ATG start codon and a *RcaI* site (respectively in bold faced letters and underlined in the sequence): hTstart (5'-CGT**CA**TGAAGCCGCTGTAGTT-3'). The 3' primer had the following sequence: hTend (5'-GAGGCGGG**TTAA**CAGTGTTTC-3'), and was designed to insert a recognition site for the *HpaI* endonuclease (underlined in the sequence shown) and contained the TAA stop codon (bold faced letters). The reaction was performed in a Perkin Elmer apparatus under the conditions described by Saiki *et al.* (1990) using as enzyme the *Pfu* polymerase (Stratagene, La Jolla, CA).

The amplified sequence was digested with *RcaI* and *HpaI* and cloned between the *NcoI* and *SmaI* restriction sites of the expression vector pTrc99A (Amann *et al.*, 1988) (Pharmacia Uppsala, Sweden), producing the vector designated pTrGadh-hT.

Site-directed mutagenesis

The point mutations were introduced in the *adh-hT* gene using an oligonucleotide-mediated PCR strategy. Glu11 and Pro242 were replaced by Lys and Ala, and two different singles and a double point mutant were constructed.

Mutant Glu11Lys was constructed by amplifying the *adh-hT* coding sequence with the hTend oligonucleotide and the upstream mutagenic primer: BSP1, 5'-CGT**CA**TGAAGCCGCTGTAGTTGAACAATTTAAGaaA-3' that allowed the insertion of the point mutation (lower case letter) and has a *RcaI* site (underlined in the sequence). The PCR product was digested with *RcaI* and *HpaI* and inserted in pTrc99A between the *NcoI* and *SmaI* restriction sites.

The Pro242Ala point mutation was introduced by amplifying

the C-terminal region of the *adh-hT* gene using the hTend primer and the 5' primer: Pra1, 5'-GTAT**CTAAG**gCAGCGT-TTC-3' which introduces the desired mutation (lower case letter) and maintains the *DdeI* site (underlined in the sequence). The PCR product was digested with *DdeI* and *HpaI* and inserted into pTrGadh-hT replacing the corresponding wild-type sequence.

The *EcoRI*–*HindIII* gene fragment containing the Pro242Ala mutation was inserted in the Glu11Lys mutant into the same restriction sites in order to generate the double mutant, Glu11-Lys/Pro242Ala, containing both substitutions in the *adh-hT* gene sequence.

The insertion of the correct point mutations in all genes was confirmed by DNA sequencing with the Sanger dideoxynucleotide termination method using the Sequenase version 2.0 kit (USB, Amersham).

Gene expression and enzyme purification

Escherichia coli RB791 (Brent and Ptashne, 1981) were transformed with the newly constructed plasmids and grown in Luria-Bertani medium supplemented with 0.25 mM ZnSO₄ with 100 µg/ml ampicillin at 37°C to cell densities corresponding to an OD₆₀₀ of 1.5. The expression was induced by the addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG, Inalco) and the cells grown in the presence of the inducer for 16 h. The cell pellet was resuspended in 10 ml of 50 mM sodium phosphate, pH 7.0, and crude extracts were prepared by disrupting the cells in a Sonicator® Ultrasonic Liquid Processor (Heat Systems-Ultrasonics Inc., NY) by 10 min pulses at 20 kHz and recovered after 1 h ultracentrifugation at 36 000 g. Purification of recombinant wild-type and mutant enzymes was carried out as described previously (Cannio *et al.*, 1994). The crude extract was heated at 60°C for 10 min and centrifuged at 5000 g at 4°C for 10 min; the supernatant was concentrated fivefold by ultrafiltration in an Amicon cell (membrane cut-off 10 000 Da), and reheated under the same conditions. The sample was then loaded onto a Matrex gel Blue-A column (Amicon) and specifically eluted using a linear gradient of NAD (0.2–2 mM).

When NAD-free preparations were required, proteins were purified loading the heat-treated extracts onto an S-200 gel filtration column (Pharmacia, 2.6×60 cm) connected to an FPLC (fast protein liquid chromatography) system (Pharmacia) under the conditions described for ADH purification from *Sulfolobus solfataricus* (SsADH) by Cannio *et al.* (1996).

ADH activity was assayed by following the reduction of NAD at 340 nm in a Cary IE recording spectrophotometer. The standard ADH assay was performed at 60°C in a mixture (1 ml) containing 25 mM sodium phosphate buffer, pH 8.0, 30 mM ethanol or 50 mM benzyl alcohol, 5 mM NAD, and the extract or the purified protein to be assayed. One unit of enzyme activity was defined as the amount of enzyme that catalyses the reduction of 1 µmol NAD per min at 60°C under the conditions described. Specific activity is expressed as units per mg of protein.

Protein concentration was determined as described by Bradford (1976) using the Bio-Rad assay kit and the bovine serum albumin (Sigma) as standard.

SDS-PAGE analysis followed the method described by Laemmli (1970) while the method of Davis (1964), using phenazine methosulfate and nitrobluetetrazolium (Sigma), was used to detect ADH activity on a non-denaturing electrophoresis 7% polyacrylamide slab gel.

Kinetic measurements

Different primary or secondary alcohols at the saturating concentration of 30 mM were tested as substrates for the wild-type and mutant enzymes under the standard conditions.

The kinetic parameters versus benzyl alcohol were determined at 60°C in 25 mM Na-phosphate buffer, pH 8.0, 10 mM NAD and benzyl alcohol concentrations ranging from 0.01 to 70 mM, while to calculate the kinetic constants towards NAD, the coenzyme concentration was varied from 0.01 to 20 mM in the same phosphate buffer containing benzyl alcohol at a concentration of 50 mM.

Aliquots of 0.3 µg of both wild-type and mutant enzymes were used in the assay mixture. All assays were made in triplicate, their linearity always observed for 5 min, and experimental data analysed and refined with the GraFit program (Leatherbarrow, 1992). NAD stability was verified by controls in which the assay mixtures were incubated in the assay conditions without adding the enzyme; no variation in absorbance was registered over 5 min.

The optimal pH values for enzyme kinetics were determined by measuring the reaction rates at 60°C in 25 mM Na-phosphate buffer in the pH range 6.5–8.4, and 25 mM glycine–NaOH in the pH range 9.0–10.0 (the pH values were measured at the assay temperature).

The temperature/activity profiles were determined by assaying the enzyme activity (0.3 µg) under the standard conditions, but gradually increasing the temperature from 35 to 75°C (5°C at a time).

Enzyme stability

Thermal resistance was investigated at 70 and 75°C. The purified enzymes (24 µg) were incubated in 25 mM sodium phosphate buffer pH 7.0 in a final volume of 0.6 ml. Aliquots (50 µl) were assayed every 5 min under standard conditions, and the residual activity measured.

The deactivation in the presence of guanidine–HCl was calculated by incubating the proteins (40 µg) at room temperature in 25 mM Na-phosphate buffer, pH 7.0 containing 0.2, 0.5 or 1 M guanidine–HCl (Sigma). The residual activity was calculated on 50 µl aliquots every hour for 16 h in a standard assay.

In order to evaluate the effect of the pH on the stability, the proteins (30 µg) were incubated for 16 h in different 1 ml 25 mM buffers with different pH values: Na-citrate pH 4.2, Na-phosphate in the pH range 6.0–8.0, and glycine–NaOH in the range 9.0–10.0. After incubation, 10 µl aliquots were assayed under the standard conditions.

Results

Gene expression and protein purification of recombinant ADHs

High level expression of wild-type and mutant ADHs was obtained in *E.coli* using an IPTG inducible system producing comparable amounts of the proteins under study. Optimal conditions for the growth and IPTG induction of *E.coli* RB791 transformants were determined according to the method described by Guagliardi *et al.* (1995).

The recombinant wild-type and mutant ADH were purified to similar homogeneity grade. Two successive thermal precipitation steps allowed the contaminant mesophilic proteins to be almost completely removed. The purified active samples were recovered after loading onto a dye–ligand affinity chromatography and specific elution with the coenzyme as judged by

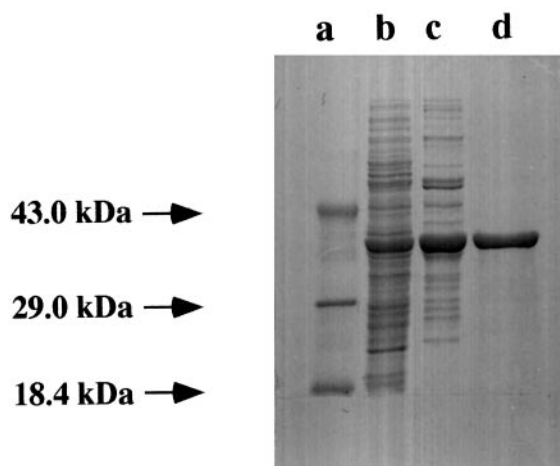


Fig. 1. SDS–PAGE analysis of purification steps for the recombinant wild-type ADH. Lane a, molecular mass standards; b, crude protein extract of recombinant *E. coli* RB791; c, protein extract after two thermal precipitation steps; d, sample after dye–ligand (Blue-A) chromatography. Identical results were obtained also for the mutant proteins.

SDS–PAGE analysis (Figure 1). Nevertheless, the mutants containing the Pro242Ala substitution were eluted earlier than the wild-type and the Glu11Lys mutant.

Spectrofluorimetric analysis revealed that, also after extensive dialysis, trace amounts of NAD were contained in the purified proteins; therefore, for studies of protein stability NAD-free preparations were obtained by purification with gel filtration chromatography. The elution profile showed the presence of two major peaks, one eluting at a volume corresponding to the molecular mass of the tetramer ADH and another eluting as an inactive dimeric form. This was also confirmed by SDS–PAGE where a single co-migrating band was observed when loading aliquots of the two denatured peaks. In any case, when Zn^{2+} ions were added to the culture medium, a greater peak corresponding to the tetramer and a higher total specific activity could be measured.

SDS–PAGE analysis revealed no difference in the molecular mass from that of the wild-type enzyme already described; moreover, the recombinant ADH-hT had the same pI, thermal stability and specific activity of the native enzyme purified from *Bacillus stearothermophilus* (Guagliardi *et al.*, 1996). Therefore, the recombinant ADH-hT was used for all the subsequent characterizations and will be referred to in this paper as the ‘wild-type’ enzyme.

After a typical purification procedure, about 40 mg of pure ADH-hT protein was obtained from 1 litre of *E.coli* culture cells. The same yields and purification were also obtained for the mutant proteins.

Gel electrophoresis under nondenaturing conditions revealed the slower electrophoretic mobility of the mutants containing the Glu11Lys substitution in comparison with the wild-type and Pro242Ala mutant, thereby confirming the ionic nature of the mutation (Figure 2).

Kinetic analysis of wild-type and mutant ADH

Wild-type and mutant ADH were tested on different substrates (Table I) with the wild-type being the more active and the double mutant the least active when ethanol is used as the substrate; otherwise, the specific activities calculated for the mutants were in all cases higher than that of the less thermostable ADH-T (Figure 3), demonstrating that the substitutions were not disruptive; moreover, the overall structure of the

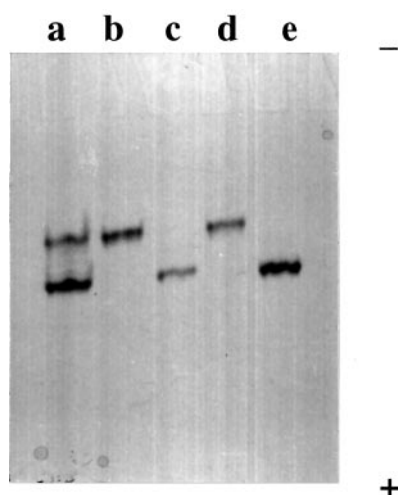


Fig. 2. Nondenaturing PAGE of wild-type ADH-hT and Glu11Lys, Pro242Ala, Glu11Lys/Pro242Ala. Proteins are detected using ADH zymography (activity staining). Lane a, ADH-hT and Glu11-Lys loaded in the same slot; b, Glu11Lys/Pro242Ala; c, Pro242-Ala; d, Glu11-Lys; e, ADH-hT.

Table I. Specific activity of wild-type and mutant ADHs

	ADH-hT	Glu11Lys	Pro242Ala	Glu11Lys/ Pro242Ala
Ethanol	300.0	265.2	172.1	148.3
Methanol	0	0	0	0
1-Buthanol	263.4	200.2	136.7	116.7
2-Buthanol	382.7	305.6	236.7	186.7
Isopropyl alcohol	292.3	280.1	213.3	157.7
Cyclohexanol	0	0	0	0
Benzyl alcohol	204.1	178.4	233.3	207.9

Values are shown as U/mg at 60°C.

molecules was not affected, as demonstrated by far-UV CD spectra registered at 25 and 60°C (data not shown).

In Table I the specific activities at 60°C of the wild-type and the three mutants for a number of different substrates are also listed. All the substrates were tested at saturating concentrations determined in previous assays. All the proteins are completely unreactive towards methanol and cyclohexanol and show a strong preference for secondary rather than primary alcohols, as also reported for all the thermophilic ADHs whose substrate specificity has been investigated (Keinan *et al.*, 1987). The mutants containing the Pro242Ala substitution display a higher catalytic efficiency towards benzyl alcohol than towards ethanol.

pH profiles of enzyme activity for wild-type ADH-hT and the mutants were essentially overlapping (optimum at pH 8.0). Similarly, for the thermal activities in the temperature range investigated, differences were detected only in the $V_{\max}$ values, whereas in all the Arrhenius plots a break-point at 50°C was shown (Figure 3).

The kinetic constants determined for the wild-type and mutants at 60°C are summarized in Table II. Due to the kind of mutations introduced, the differences in the kinetic constants between ADH-hT and the mutants do not vary significantly. In any case, as previously reported, it can be noted that the mutants containing the Pro242Ala substitution convert the benzyl alcohol more efficiently than ADH-hT (higher k_{cat}/K_m

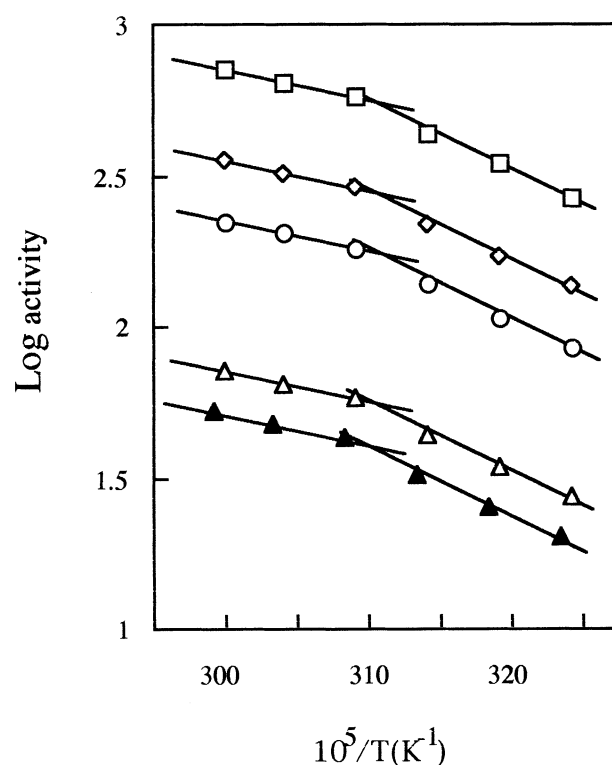


Fig. 3. Thermal activity of recombinant ADH-hT (□), Glu11Lys (◇), Pro242Ala (○), Glu11Lys/Pro242Ala (△) and native wild-type ADH-T (▲). Log activities are calculated as nmol/min and plotted against the reciprocal of temperature values (1/K).

values) even if the K_m values calculated against benzyl alcohol are quite similar for all the enzymes.

The kinetic parameters determined for the NAD coenzyme revealed that the Pro242Ala substitution could affect the catalytic efficiency by increasing the affinity (lower K_m) towards the coenzyme.

Enzyme stability

The comparative deactivation of wild-type and mutant enzymes in the presence of heat and guanidinium-HCl was investigated in order to study the effect of the mutations introduced on protein stability.

The resistance to heating was determined at 70 and 75°C. The wild-type and mutant enzymes retained similar activity after incubation at 70°C. Their behaviour differed at 75°C as demonstrated by the dependence of the residual enzyme activity, shown in Figure 4, as a function of the incubation time: the recombinant ADH-hT still retains 75% activity after 30 min, while 50% deactivation occurs in Glu11Lys, Pro242Ala and ADH Glu11Lys/Pro242Ala mutants after 30, 26 and 18 min, respectively.

Their behaviour also differs in the presence of 0.5 M guanidinium-HCl; in fact, while 50% residual activity is measured for ADH-hT after 12 h incubation, the same decrease in activity occurs in the double mutant after only 6 h. Lower or higher guanidinium-HCl concentrations gave less measurable differences on protein stability.

The effect of pH on the activity was quite similar for wild-type and mutant proteins which were found to be inactive after 16 h incubation in Na-citrate pH 4.2 and retained 50% activity when incubated at pH 10. The highest activities were recovered after incubation at pH 7.5.

Table II. Kinetic constants at 60°C of wild-type and mutant enzymes

	k_{cat} (s ⁻¹)		K_{m} (mM)		$k_{\text{cat}}/K_{\text{m}}$ (s ⁻¹ mM ⁻¹)	
	Benzyl alcohol	NAD	Benzyl alcohol	NAD	Benzyl alcohol	NAD
ADH-hT	720.8±3.2	700.6±6.2	10.2±0.5	1.5±0.17	70.7	467.1
Glu11Lys	575.6±3.4	550.7±2.6	12.1±0.6	1.2±0.08	47.6	458.9
Pro242Ala	888.1±5.2	834.0±4.7	14.2±0.8	0.7±0.07	62.5	1191.4
Glu11Lys/ Pro242Ala	651.9±5.8	675.4±2.2	15.5±0.8	1.0±0.04	42	675.4

Discussion

In the framework of a project aiming at studying oxidoreductases from thermophiles, we assessed the expression at high levels of ADH-hT from *Bacillus stearothermophilus* (strain LLD-R) in *E.coli* by optimizing both the growth and the induction times for the transformed cells. The purification to homogeneity of the recombinant protein was easily obtained by enriching the extract of *E.coli* in the heat stable bacillar fraction by two simple thermal precipitation steps and subsequent dye–ligand affinity or gel filtration chromatography. The expression system and the purification procedure chosen allowed us to obtain up to 25 mg of homogeneous protein from a 1 litre scale culture of transformed *E.coli*. When Zn²⁺ ions were added to the culture medium the amount of purified active protein reached up to 40 mg. This confirms the structural role of Zn²⁺ ions in guaranteeing the correct folding and the conformational stability of the native quaternary structure. The recombinant ADH-hT retains all the native enzyme properties indicating that thermophilic enzymes are intrinsically thermostable.

Although it is now ascertained that there is no unique and general definition for protein stability (Vieille and Zeikus, 1996), the direct comparison of thermophilic proteins with different thermal features can help in clarifying the role of single amino acids in the stability of thermophilic enzymes. In fact, when the ADH-hT primary structure had been aligned with that of the less thermostable ADH-T from the more well-characterised NCA 1503 strain of *B.stearothermophilus*, three significant substitutions were pointed out, presumed to be responsible for the different thermal features of the two enzymes compared (Cannio *et al.*, 1994). In order to validate the role of the salt bridges and the reduction of the entropy of unfolding hypothesised in our model system, the Glu11 and Pro242 residues were substituted by the amino acids present in the same position (Lys and Ala) in the less thermostable ADH-T where these stabilizing effects seemed to be absent.

The *adh-hT* gene was mutated at the desired positions, expressed, and all the corresponding proteins purified under the same conditions, so that the differences in chemical, physical and kinetic parameters could be ascribed only to the substitutions introduced and not to the post-translational modifications of the host system.

The point mutations were found to progressively decrease the stability towards denaturing agents such as heat and guanidinium hydrochloride, rendering the proteins progressively more similar to ADH-T with the proline replacement showing a more sensible effect on thermoresistance when compared with the elimination of the salt bridge.

Substrate specificity and the thermal activity were studied, and the kinetic parameters for benzyl alcohol and NAD coenzyme calculated, to exploit the effect of the mutations also on the enzyme activities. The catalytic efficiency of the

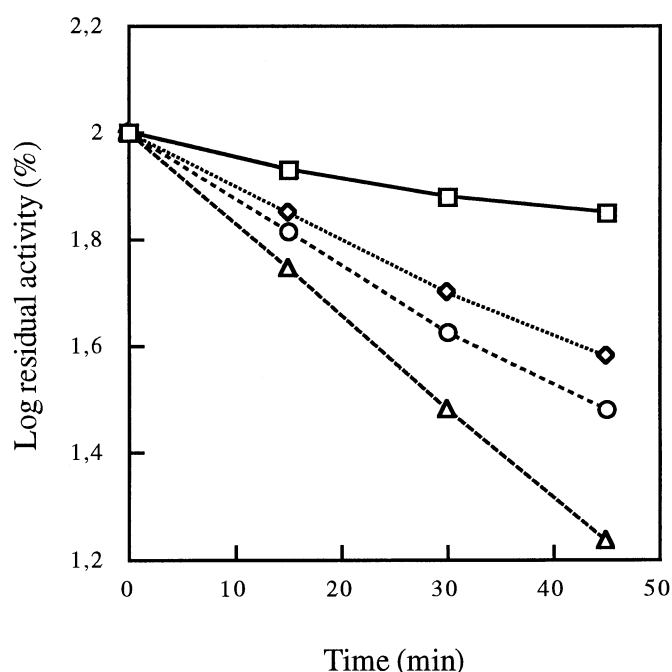


Fig. 4. Thermal inactivation of ADH-hT (□), Glu11Lys (◇), Pro242Ala (○) and Glu11Lys/Pro242Ala (△). Log activities are calculated as U/mg and plotted against the time of incubation at 75°C.

mutants still remained higher than that of ADH-T (Guagliardi *et al.*, 1996), while the unaltered thermal activity and pH optimum confirmed that the mutations introduced did not affect the general catalytic mechanism even if they were responsible for a modified catalytic efficiency. Moreover, as predicted in our hypothesis, they did not introduce any radical change or dramatic destabilizing effect in the structure as reported in other analogous studies (de Prat Gay *et al.*, 1994; Herning *et al.*, 1992; Pappenberger *et al.*, 1997). Otherwise, the fact that ADH-hT and ADH-T are evolutionary very closed proteins supports the hypothesis that the replacements under investigation could represent intermediate steps in the evolution from a less to a more stable structure. In fact, the ADH-T protein still remains the least stable; none of the mutations introduced in ADH-hT was able to decrease the features below those relative to ADH-T.

All the mutants showed the same broad substrate specificity over the alcohols tested: interestingly, the catalytic efficiency towards benzyl alcohol of the mutants carrying the Pro242Ala substitution was higher than that calculated towards ethanol; an inverse behaviour was observed for the wild-type enzyme. This result suggests that the structural rigidity of the Rossmann fold conferred by the proline reduces the rate of access of bulky substrates such as benzyl alcohol to the catalytic site and hence the catalytic oxidation. On the other hand, the

substitution of the proline with the smaller alanine almost doubles the affinity of the enzyme towards NAD. This fact is not so surprising considering that the mutation introduced occurs in the coenzyme binding domain and can affect NAD binding and release. In fact the similar K_m values calculated towards benzyl alcohol for the wild-type and the mutants, and the higher k_{cat} values calculated for the Pro242Ala and the Glu11Lys/Pro242Ala mutants show that all the enzymes could bind the aromatic alcohol with the same affinity, but that the higher affinity towards the coenzyme for the mutants containing the Pro242Ala substitution could accelerate the slow step of the catalytic reaction that, according to the general mechanism of action of other ADHs, is the dissociation of the enzyme–NADH complex (Shearer *et al.*, 1993).

In conclusion, the direct comparison of proteins with increasing thermoresistance, thanks to the higher degree of similarity, constitutes a simpler and more effective method to elucidate the role of single amino acid substitutions in enzyme stability and catalysis. Moreover, this approach demonstrates that the changing of structural motifs not located in the catalytic site can nevertheless direct the catalytic specificity towards different substrates of new tailored enzymes, with no loss in the overall efficiency.

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