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RESEARCH ARTICLE

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Inactive and temperature-sensitive folding mutants of aldehyde dehydrogenase of *Escherichia coli*

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Summary Aldehyde dehydrogenase, encoded by the *aldA* gene in *Escherichia coli*, is inactive in nitrosoguanidine induced mutant strain ECL40 and temperature-sensitive in spontaneous mutant strain JA104. Both mutants were proven, by complementation experiments, to have a functional *aldA* regulator and promoter. In spite of no immunodetection of the *aldA* product, its specific transcript was present in the mutant extracts. It was subsequently proven that the immunodetection of aldehyde dehydrogenase in these mutants required denaturation, revealing that cells lacking the enzyme activity had the inactive protein in their extracts. Thus, the mutations seemed to affect the protein conformation. The temperature-sensitive aldehyde dehydrogenase did not show, neither in vivo nor in vitro, a different thermal stability compared to the wild type enzyme. In this temperature-sensitive strain, the recovery of active aldehyde dehydrogenase, in the presence of rifampicin but not of chloramphenicol, when cells grown at 37°C were shifted to 30°C indicated that this mutation affected the folding process of the protein at the restrictive temperature. Sequencing of the two mutant *aldA* corresponding genes determined a single amino acid change of Pro to Leu at position 182 for strain ECL40, and of Val to Met at position 145 for strain JA104. These mutations were thought to possibly promote changes in the local flexibility in the first case, and to perturb the packing of residues by steric hindrance in the second case.

Key words *Escherichia coli* · Folding-mutants · Temperature-sensitive · Aldehyde dehydrogenase · *aldA* Gene mutations

Introduction

The gene *aldA* maps at min 32 [5, 12] of the *Escherichia coli* chromosome. It has been sequenced (GenBank accession number M64541) and reported to encode an aldehyde dehydrogenase (ALDH) of 479 amino acids [18] acting on a broad spectrum of substrates. The deduced amino acid sequence displays important homologies with other aldehyde dehydrogenases that have highly conserved regions, such as the coenzyme binding domain and other regions of unknown function. The most conserved regions are confined to the central part of the protein with several identical peptide sequences, whereas the N-terminal part is poorly conserved [18]. ALDH activity is absent under anaerobic conditions, as its encoding gene *aldA* is repressed by the Arc aerobic-anaerobic control system [22]. Gene *aldA* is also under the control of two regulatory proteins, which recognize two types of inducers, hydroxyaldehydes and 2-oxoglutarate, and under the control of catabolite repression protein [22]. Initially ALDH was assigned the role of oxidizing to L-lactate the L-lactaldehyde [34] formed in the metabolism of L-fucose [13] and L-rhamnose [4]. Further studies demonstrated that it was also involved in the oxidation of glycolaldehyde, formed in the metabolism of different pentoses [21], and in the metabolism of ethylene glycol in mutant cells adapted to grow on this non-natural carbon source [10].

A mutant defective in ALDH activity (strain ECL40) was isolated after nitrosoguanidine mutagenesis by Sridhara and Wu [34]. This mutant was shown not to be able to react with specific antibodies, and was supposed not to synthesize the enzyme protein [4]. Subsequently, Caballero et al. [11] isolated without mutagenesis, another ALDH defective mutant (strain JA104): a temperature-sensitive mutant deprived of ALDH activity when grown at restrictive temperature (37°C) but displaying activity at permissive temperature (30°C). As for strain ECL40, the temperature-sensitive mutant strain JA104 had no protein cross-reacting with ALDH antibodies in the extracts of cells grown at 37°C, whereas in extracts of cells grown at 30°C, immunodetection yielded sizable amounts of specific ALDH protein [5].

Taking into account the absence of immunological detection of aldehyde dehydrogenase in strains ECL40 and JA104 (grown at restrictive temperature), and the equal thermal stability of the wild type and temperature-sensitive enzymes, it was proposed that these strains had a defective expression of *aldA* gene [4, 5]. However, in this report we characterize these two mutations, and prove them to be structural mutations affecting the folding of the protein.

Materials and methods

Bacterial strains Wild type *E. coli* used in this study was strain ECL1 [23] also known as E15 [3]. The two aldehyde dehydrogenase mutant strains studied were: strain ECL40 [34] and the temperature-sensitive mutant, strain JA104 [11]. This temperature-sensitive mutation of strain JA104 was transduced into the same genetic background of strain ECL40 and transductants (strain JA114) selected by its recovered ability to grow on L-1,2-propanediol at 30°C but not at 37°C. Strain ECL40 and JA114 were both made *recA* by a Tn*10* insertion generating strains JA111 and JA115 respectively. Strains MC1061 [26] and RYC1000 [22] were used as transduction recipients in complementation experiments with the fusion bearing plasmids.

Growth conditions Cells were grown aerobically on Luria-Bertani broth or minimal medium as described previously [8]. For growth on minimal medium, carbon sources were added to a basal inorganic medium in the following concentrations: glucose 10 mM, L-rhamnose 10 mM, casein acid hydrolysate 0.5%. Anaerobically, carbon sources were added to minimal medium at double concentration [8]. When necessary, the following antibiotics were added to the medium at the concentrations indicated: ampicillin 100 µg/ml, kanamycin 25 µg/ml, streptomycin 30 µg/ml and tetracycline 12.5 µg/ml. In order to inhibit transcription or translation, rifampicin and chloramphenicol were respectively added to a concentration of 170 µg/ml. Isopropyl β-D-thiogalactoside and 5-bromo-4-chloro-3-indolyl-β-D-galactoside were used at 10 µg/ml and 30 µg/ml respectively.

Preparation of cell extracts and enzyme assay The cells were harvested at the end of the exponential phase and the cell extract was prepared as described previously [9] in 10 mM Tris-HCl buffer (pH 7.0).

The aldehyde dehydrogenase activity was assayed spectrophotometrically (at 340 nm) by monitoring the increased rate of NADH formation at 25°C. Since this enzyme was also reported to be responsible for the conversion of glycolaldehyde to glycolate [11] glycolaldehyde was routinely used instead of lactaldehyde since the latter is not commercially available. The assay mixture (1 ml) consisted of 1 mM lactaldehyde or glycolaldehyde, 100 mM sodium glycine buffer (pH 9.5), and 2.5 mM NAD.

The β-galactosidase activity in strains grown under specified conditions was assayed as described by Miller, and the values are reported in the units defined by this author [27]. The results given, both for the ALDH and for β-galactosidase, are the mean of three different experiments with a sd < 15%.

The protein concentration in cell extracts was determined by the method of Lowry et al. [25] with bovine serum albumin as the standard.

Immunodetection procedures and Western Blot Antisera against ALDH were raised in New Zealand White rabbits as described previously [5] using as antigen the enzyme purified according to Baldomà and Aguilar [4]. Immunoelectrophoresis was performed as previously described by Laurell [20] in 1% agarose.

20–50 µg of total protein cell extracts were subjected to 12% PAGE (non denaturing) or SDS-PAGE (denaturing) in a mini-protean (Bio Rad) following the manufacturer's instructions. Proteins were then electrotransferred in a Mini trans blot (Bio Rad) to nitrocellulose filters (Immobilon Millipore) and treatments with antisera were performed as described previously [2]. The specific protein bands were stained with diaminobenzidine and peroxidase development reagent, and total protein bands were stained with Coomassie Brilliant Blue in gels or with amido-black in filters.

DNA manipulation Bacterial genomic DNA was obtained following Silhavy et al. [32]. Plasmid DNA was routinely prepared by the boiling method [19]. For large scale preparation, a crude DNA sample was subjected to purification on a column (Qiagen GmbH, Düsseldorf, Germany). DNA manipulations were performed essentially as described by Sambrook et al. [30]. The DNA sequence was determined by using the dideoxy-chain termination procedure of Sanger et al. [31], with double stranded plasmid DNA as the template. We resolved sequencing gel compressions as described previously [18].

Transcriptional fusions were constructed by inserting the DNA fragments into plasmid pRS550 as indicated by Simons et al. [33]. Single copy fusions were obtained by transferring the plasmid fusions into λRS45 by homologous recombination and subsequent infection of strain RYC1000 with the lysate to isolate the single copy fusion lysogens [33].

Amplification of DNA. PCR reactions were performed in a total volume of 100 µl; the mixture contained 50 ng of *Hin*dIII totally digested genomic DNA, 0.2 mM dNTP, 1× reaction buffer as supplied with *Taq* polymerase (Bioprobe Systems), 2.5 units of *Taq* polymerase and 25 pmol of each primer. Reactions were set in an ice bath and the DNA was heat-denatured (10 min at 95°C) prior to being added to the reaction tubes. The reaction was allowed to proceed for 35 cycles which consisted of: 60 s at 94°C, 90 s at 55°C and 210 s at 72°C. Finally the reaction was incubated 10 min a 72°C. PCR products were analyzed after size separation in 1% agarose gels by ethidium bromide staining. Sequences of the 18-mer amplimers used in the amplification of the *aldA* region of mutants were 5′ A G G C T T G C G G A G T G G C G G 3′ and 5′ C G A C T G G T G T T G G G T C G A 3′.

RNA preparation and Northern blot experiments For the preparation of total RNA, cells of a 25-ml culture grown to A_{650} of 0.5 were collected by centrifugation at 5000× *g* for 10 min and processed as described by Belasco et al. [7]. Northern-blot hybridization was performed with each RNA sample (10 µg) following the procedure described previously [28]. The probe was [^{32}P] labeled by the random primed method [30] using as template a 500 bp (*Pst*I-*Bam*HI) *aldA* internal fragment [18].

Results

Expression of ALDH in mutant strains JA111 and JA115 On the basis of the absence of ALDH activity and protein in mutant ECL40 and in temperature-sensitive JA104 grown at restrictive temperature, it was proposed that these strains had a defective regulation of *aldA* gene expression [4, 5]. However, their derivatives strain JA111 and JA115 had a functional activator, as transcriptional fusions of *lacZ* gene with wild type *aldA* promoter showed activation by the *aldA* regulator protein of the mutant cells. In addition, the expression of b-galactosidase from the *aldA* promoters of the mutant cells fused to *lacZ* did not support the hypothesis of a mutation affecting the transcriptional activity of these promoters (not shown).

The active function of the *aldA* regulator protein and promoter in mutant strains JA111 and JA115 led us to further study the expression of *aldA* gene in these mutant strains. Transcriptional activities of *aldA* gene of wild type and mutant strains shown by the expression of the corresponding promoter fusions to *lacZ* were confirmed by Northern analyses. Total RNA was prepared from strain ECL1, JA111, and JA115 grown aerobically on the inducing sugar L-rhamnose at 30°C and 37°C. A culture of strain ECL1 in anaerobiosis was carried out in parallel as negative control. Specific *aldA* transcripts were determined by hybridization of the electrophoresed RNA with a labeled probe of the *aldA* structural gene. An apparent transcript was detected for all, wild type and mutant strains, either at 30°C or at 37°C, whereas no band corresponding to *aldA* transcript was detected when the gene was repressed by growth of cells in the absence of oxygen (not shown).

Inconsistencies between the presence of specific RNA and absence of ALDH activity and protein in strain JA111 or strain JA115 grown at 37°C (not shown) suggested a post-transcriptional effect of the corresponding mutations. For this reason, we analyzed the product of the *aldA* transcripts, revealed by immunodetection as indicated above. The immunoprecipitation reaction was studied for the different ALDH preparations in a denaturing and non-denaturing conditions. Extracts of strain ECL1, JA111, and JA115 grown aerobically on rhamnose at 30°C and 37°C were electrophoresed with and without SDS. The gels were Western blotted, reacted with antisera, washed, and the immunoprecipitated bands stained with diaminobencidine and peroxidase. The gel run under denaturing conditions displayed a band of immunoprecipitate for each of the extracts tested indicating the presence of ALDH protein (Fig. 1). In the case of the gel run in non-denaturing conditions, the native protein of strain JA111 was not detectable

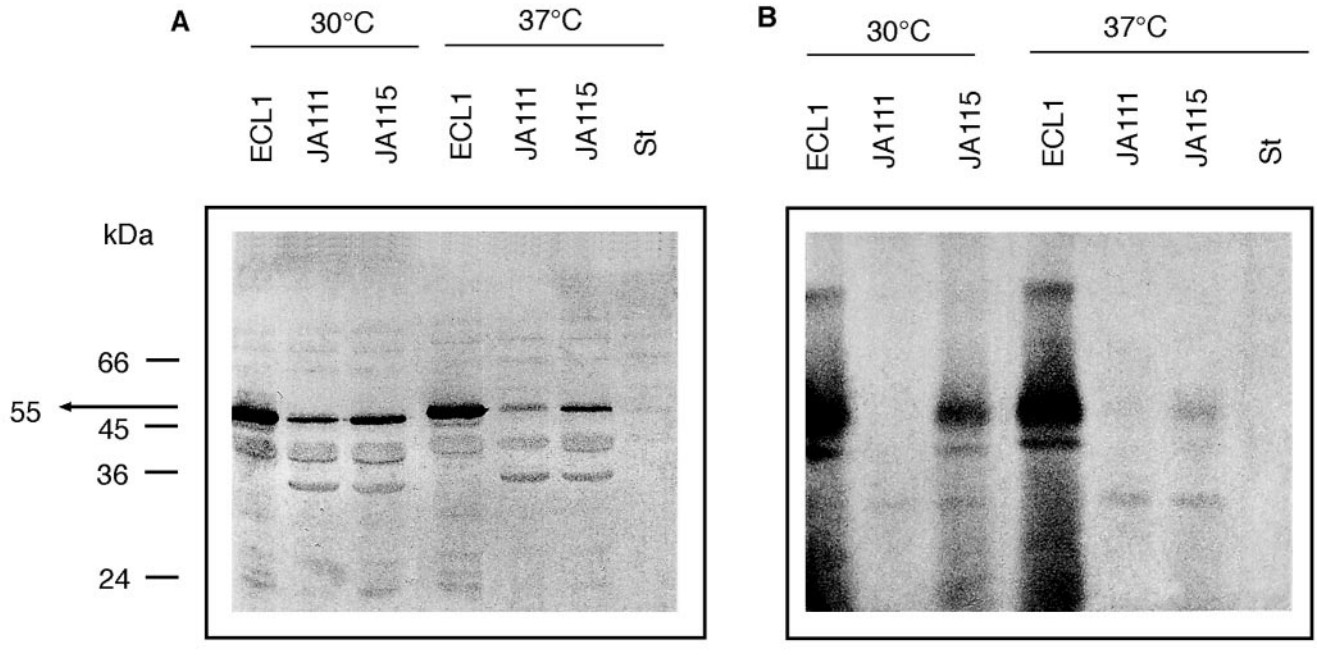

Fig. 1 Western blots of extracts of strains ECL1, JA111 and JA115 grown aerobically on rhamnose. Extracts of *Salmonella typhimurium* LT2 which lack aldehyde dehydrogenase [6] were used as negative control (labeled st). The extract proteins were run in PAGE under denaturing conditions (panel A) or non-denaturing conditions (panel B) and the ALDH specific bands revealed by immunodetection with specific antibodies and staining with diaminobenzidine and peroxidase development reagent. Size of the proteins in kDa according to markers used in the experiment and ALDH (55 kDa) position are indicated

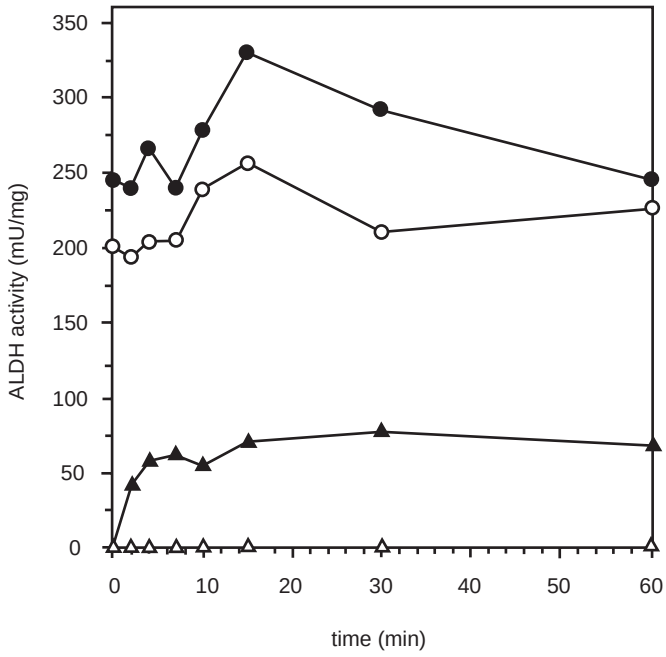

Fig. 2 Time course of ALDH activity of strain ECL1 (circles) and JA115 (triangles) grown aerobically on rhamnose at 37°C and shifted to 30°C (time 0) with either of the following additions at the time of the shift: chloramphenicol (open symbols), rifampicin (closed symbols)

by immunoprecipitation in the cells grown either at 30°C or 37°C. In contrast, the native ALDH of temperature-sensitive mutant strain JA115 was not detectable by immunoprecipitation when the cells were grown at the restrictive temperature of 37°C, but gave a clear immunoprecipitate when the cells were grown at 30°C (Fig. 1).

Thermal stability of native ALDH from temperature-sensitive strain JA115 was determined in an experiment in which cells of this strain were induced to synthesize ALDH by growth on rhamnose aerobically at 30°C, and then the cells shifted to 37°C in the presence of chloramphenicol. An experiment with strain ECL1 was run in parallel for comparison. At the indicated times samples were withdrawn for enzyme activity and Western blot immunodetection assays. No change in enzyme activity or in immunodetection was apparent after 60 min of incubation at 37°C, thus indicating a good stability of ALDH protein at the restrictive temperature (not shown).

Thermal sensitivity affects the folding of the polypeptide The reciprocal culture shift from 37°C to 30°C was performed for strain JA115 and the cells incubated after the shift in the presence either of chloramphenicol, as inhibitor of translation, or rifampicin, as inhibitor of transcription. The cells grown aerobically on rhamnose at 37°C, hence deprived of native active ALDH, displayed no activity after being incubated at 30°C in the presence of chloramphenicol for as long as 60 min. In contrast, if the culture was shifted to the permissive temperature in the presence of rifampicin, a rapid increase in the ALDH activity was detected in the first 15 to 20 min. No significant differences in ALDH activity were observed when cells of strain ECL1 were subjected to the same culture conditions shift (Fig. 2).

Amino acid changes in ALDH of mutant strains JA111 and JA115 To clone the *aldA* gene of mutant strain JA111 and JA115 a fragment was PCR-amplified from a *Hind*III totally digested genomic DNA, as indicated above. Subsequently *Eco*RV digestion and ligation to the polylinker of the Bluescript plasmid were performed (See physical map in reference [18]). The sequence was determined from T3 and T7 primers and different internal synthetic oligomers. Two independent amplifications for each mutant strain were cloned and sequenced to avoid the identification of mutations due to possible PCR errors. The sequence was determined twice for the two strands of each cloned *aldA* gene.

The wild type *aldA* sequence reported by Hidalgo et al. [18] was compared to the sequences obtained in this work for the mutant *aldA* genes and the nucleotide and amino acid changes determined. The mutation in strain ECL40 or its derivative strain JA111 was a transition C to T that resulted in a change of the proline (CCA) at position 182 by a leucine (CTA) whereas the mutation in strain JA104 or its corresponding derivative, strain JA115, was a transition G to A that changed the valine (GTG) at position of 145 by a methionine (ATG) (Fig. 3).

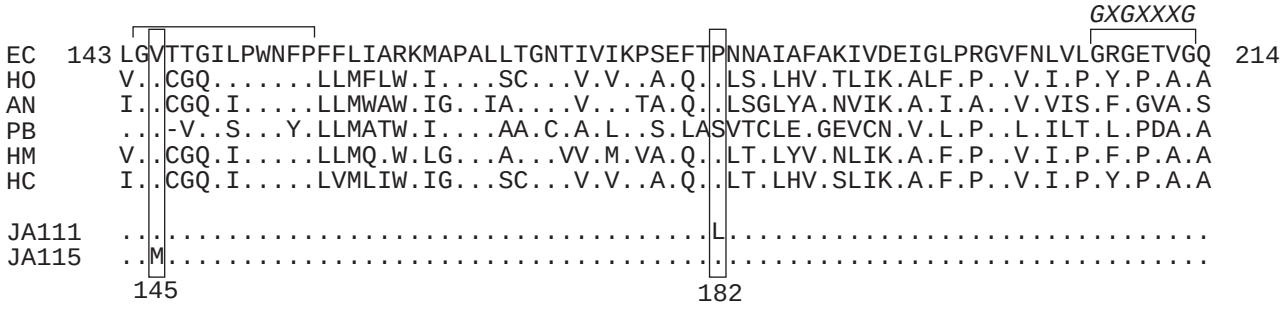

Fig. 3 Alignment of several ALDH sequences encompassing the amino acid positions of either of the two mutations. From top to bottom: *Escherichia coli* K-12, horse cytoplasm, *Aspergillus niger*, betaine aldehyde of *Spinacia oleracea*, human liver mitochondria and human liver cytoplasm. Numbers refer to the amino acid of the *E. coli* protein. Dots indicate amino acid identity with the corresponding amino acid in *E. coli* aldehyde dehydrogenase. A conserved dodecapeptide and the consensus for coenzyme binding are overlined and the conserved positions mutated are boxed

To confirm the specific mutation identified in each *aldA* gene mutant, two oligomers of the wild type sequence in which the 3´-end corresponded to the position of the mutated nucleotide were constructed. These oligomers were then used with partner oligomers in a PCR amplification of part of wild type or each of the corresponding mutant *aldA* gene DNA. A product of amplification was found when wild type DNA but not the mutant DNA was the template, thus indicating that the mutant templates had the 3´-end mismatching.

Discussion

Analysis of the immunologic recognition of ALDH under different conditions and enzyme preparations showed that in mutant extracts the undetectable protein was revealed by applying denaturing conditions to the processing of the protein. This seems to indicate a conformation-dependence of the immunodetection. Given that the antibodies against ALDH are polyclonal, the changes in conformation brought about by the altered folding in the mutant enzymes have to be very large as they affect all the epitopes involved in the antibody-protein recognition. This altered folding, as a consequence of the mutation in the enzyme of strain JA111, is unavoidable in the culture conditions used.

Experiments in which the cells of the temperature-sensitive strain JA115 were shifted from 37°C to 30°C confirmed the folding differences in ALDH. The recovery of the active ALDH in the first 15 min of incubation at 30°C when the shift was made in the presence of rifampicin as an inhibitor of the transcription initiation, indicate that mutant ALDH synthesis and folding, which was impaired at 37°C, occurred normally at 30°C. Under these conditions, de novo formation of active ALDH was directed by translation of preexisting transcripts formed before the shift. In accordance with these observations, when this shift was made in the presence of chloramphenicol as an inhibitor of protein synthesis, no active ALDH was detected, even after 60 min of incubation.

A more precise characterization of the nature of the *aldA* gene mutations of strain JA111 and JA115 was obtained by direct sequencing of both mutant *aldA* genes. The single amino acid change in each ALDH mutant was in positions 145 (Val) and 182 (Pro), two highly conserved positions among the different aldehyde dehydrogenases of several species (Fig. 3), probably corresponding to fundamental locations of their side chains for folding. Alignment by Hempel et al. [17] of 16 NAD and or NADP-linked aldehyde dehydrogenases showed that 11 out of 23 invariant residues are glycine and 3 are proline, indicating mutational restraint against alteration of peptide-chain bending points. In addition, other 66 positions show high conservation of residues, mostly hydrophobic including several valines. Among these, 10 occur in predicted beta-strands (as valine145 in ALDH), suggesting important interior-packing interactions. The first structure of an aldehyde dehydrogenase reported recently [24] confirmed these predictions. These authors suggest that all classes of aldehyde dehydrogenases have similar structural folds, despite differences in sequence, modes of aggregation and substrate specificity.

The mutation in strain JA111 leads to an anomalous conformation due to an altered folding. This different enzyme conformation not only inactivated the enzyme but also hid the epitope(s) recognized by polyclonal antibodies against the wild type enzyme (see above). The change of Proline 182 to Leucine affect a position important in the folding process, but not necessarily in the stability of the mature protein. According to both Garnier [15] and GGBSM [16] methods for structure prediction, the proline caused a turn and coil conformation, presumably playing an important role in the folding kinetics. When leucine is at position 182, the turn and coil conformation becomes a helical conformation with a probability of bend occurrence one order of magnitude lower as compared to the probability of 4.5×10^{4} corresponding to the proline containing protein. The particular role of x-proline peptide bond cis/trans isomerization in chain-folding initiation sites and the kinetics of folding of several proteins such as bovine pancreatic ribonuclease A [14], or staphylococcal nuclease [29] has been well documented.

Temperature-sensitive folding mutant proteins are characterized by being synthesized in an active conformation at permissive but not at restrictive temperatures. Nevertheless, the fact that in this mutants, the protein synthesized and matured at permissive temperature is functional at the restrictive temperature, indicates that it is the folding process of the protein that is temperature-sensitive. Baldomà and Aguilar [5] had previously shown that no difference could be detected in the thermostability of ALDH purified from temperature-sensitive strain JA104 or wild type strain. In the present work, this in vitro result was confirmed in vivo for strain JA115 (the strain JA104 derivative) by shifting a culture grown at 30°C, hence with active ALDH, to 37°C and monitoring the maintenance of ALDH level by enzyme activity and immunodetection as conformational changes indicator.

The mutant sites therefore represent positions in the sequence where the amino acid side chains play crucial roles for the stability of folding intermediates rather than contributing significantly to the stability of the protein. In this context, the temperature-sensitive folding strain JA115 mutation of valine145 to methionine may possibly perturb the packing of hydrophobic residues in an interior pocket, as has been proposed for other protein models [1].

In the characterization of these two folding mutants independently isolated we found one showing a change in the local flexibility leading to a temperature-independent change in the folding process (strain JA111), and another with a steric hindrance leading to a temperature-sensitive formation of folding initiation sites, or folding intermediates (strain JA115).

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