Drosophila alcohol dehydrogenase: evaluation of Ser^{139} site-directed mutants

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Abstract ***Drosophila*** **alcohol dehydrogenase (DADH) belongs to the large and highly heterogeneous (15–30% residue identity) short-chain dehydrogenase/reductase family (SDR). It is the only reported member that oxidizes mainly ethanol and 2-propanol among other alcohols. To confirm the role of Ser^{139} we constructed two site-directed mutants, Ser^{139}Ala and Ser^{139}Cys, which show no enzymatic activity. Molecular replacement and data from crystallographically refined 3D structures confirm the position of Ser^{139}, whose hydroxyl group faces the cleft of the presumed catalytic pocket, very close to Tyr^{152} and Lys^{156}. Thus, consistent with the constitution of the catalytic triad of other SDR, our results suggest that Ser^{139} of DADH is directly involved in the catalytic reaction.**

Key words: Site-directed-mutagenesis; Alcohol dehydrogenase; Short-chain dehydrogenase/reductase; Catalytic triad; *Drosophila*

1. Introduction

Drosophila alcohol dehydrogenase (DADH, alcohol, NAD$^+$ oxidoreductase, E.C. 1.1.1.1) catalyzes the oxidation of alcohols, mainly ethanol and 2-propanol, to the corresponding aldehydes and ketones [1]. This enzyme, active as a Zn-free dimer composed of identical subunits of 253 to 255 amino acids, plays a key role in the detoxification and metabolic assimilation of alcohols generated by microbial fermentation in the feeding niches exploited by *Drosophila*. DADH is a short-chain dehydrogenase/reductase (SDR) [2], an extended protein family, whose members show highly diverse functions, structure, subunit number and tissue distribution. Many primary structures for DADH have been reported, 57 sequences belonging to 45 *Drosophila* species, and the refined 3D structure of DADH is soon available [3].

Involvement of Tyr^{152} and Lys^{156} in the substrate-interactive domain of DADH was predicted in 1981 [4], later confirmed by biochemical data [5], chemical modification [6,7] and site-directed-mutagenesis [8–10]. In this paper we focus on position Ser^{139} whose fundamental importance in catalysis is also stressed in most SDR enzymes. Actually, from the four available SDR 3-D structures [11–16], these three amino acids have been referred to as the SDR 'catalytic triad'. To evaluate the role of Ser^{139} in catalysis in DADH, two site-directed mutants, Ser^{139}Ala and Ser^{139}Cys, were expressed in *E. coli*, purified and analyzed for enzymatic activities. Meanwhile, based on our crystallographic data, a segment containing the Ser^{139}-Tyr^{152}-Lys^{156} triad of DADH has been superimposed on the 3α,20β-HSDH, DHPR and *E. coli* uridine diphosphogalactose 4-epimerase (UDP) and the Cα least square distances were calculated. Overall, our data support the predicted involvement of Ser^{139} in catalysis and suggest similarities in the reaction mechanism of DADH with the reported SDR enzymes.

2. Material and methods

2.1. Site-directed mutagenesis and cloning of the Adh genes

Drosophila melanogaster ADH mutants were constructed from the wild-type gene cloned in the expression vector pKK^{223}-3 [9]. Mutant Ser^{139}Cys was synthesized by a two-step PCR [17] using 5′-ACATTGGATGCGTCACTGGA-3′ as mutagenic primer. Flanking primers were 5′-AAGAATTCACCATGTCGTTT-3′, which introduced an *Eco*RI site before the ATG codon and 5′-CCAAGCTTAGATGCCGGAGTCC-3′, which introduced a *Hin*dIII site after the stop codon. First PCR was performed in a final volume of 100 μl, containing 10 ng of template, 40 pmols of each primer, 200 μM of dNTPs and 2 u of Vent polymerase (New England Biolabs). Samples were kept at 94°C for 120 s and then 30 cycles were run in the following conditions: 60 s at 94°C, 60 s at 55°C, 60 s at 72°C. Products were purified after electrophoresis on 1.5% agarose gels. 30 cycles of the second PCR were performed at 55°C for the annealing step and in the previous conditions for the denaturation and elongation steps. The products of the second PCR were precipitated, digested with *Eco*RI and *Hin*dIII and cloned in the pKK223-3 expression vector. Mutant Ser^{139}Ala was generated according [18], using as mutagenic primer 5′-ACATTGGAGCCGTCACTGGA-3′ and as flanking primers the same as those for the Ser^{139}Cys gene construction. This mutagenesis method is performed in three PCR steps by sequentially adding the primers. During all PCR steps conditions were 60 s at 94°C, 60 s at 55°C and 60 s at 72°C. The first 10 cycles of the amplification were performed in a tube containing 10 ng of template, 20 pmols of the mutagenic and the downstream primers, 100 μM of dNTPs and 2 u of Vent polymerase. Then, 50 pmols of the upstream primer were added and the sample was amplified for a further 10 cycles. Finally, after adding 50 pmols of the downstream primer, the tube was subjected to the last 10 cycles. The PCR product was digested by *Eco*RI and *Hin*dIII and cloned in pKK223-3. Transformed *E. coli* JM105 cells were first screened for the mutant gene forms by a *Bam*HI digestion of their plasmid DNA, taking advantage of the loss of this restriction site when the TCC serine codon was replaced by either a TGC cysteine or a GCC alanine codon. The ADH coding region of positive clones was verified by sequencing of double-stranded DNA, using [α-^{35}S]dATP as radioactive precursor and the Pharmacia Sequencing Kit.

2.2. Expression and purification of ADH mutants

10 ml of overnight cultures of the transformed *E. coli* cells in LB-ampicillin were diluted to 100 ml with fresh medium and, after an additional 1 h (O.D. 0.6) and IPTG addition to 1 mM, cultures were incubated at 37°C for 3 h. Cells were harvested, washed twice in 20 mM Tris-HCl pH 8.6 and resuspended in 5 ml of 20 mM Tris-HCl pH 8.6 supplemented with 5% 2-propanol and 1 mM DTT. Activity assays, SDS-PAGE, immunoblotting and ADH purification were carried out at 4°C using the crude supernatant obtained after cell disruption by sonication and centrifugation for 15 min. To purify the enzymes, bacterial protein extract was concentrated to 700 μl with Centriprep Concentrators (Amicon) with a cut-off of 10 kDa. 500 μl

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of the concentrated solution was separated on FPLC MonoQ (Pharmacia) in 20 mM Tris-HCl pH 8.6, supplemented with 5% 2-propanol and 1 mM DTT. The eluent contained mainly ADH, while most of the *E. coli* proteins remained bound to the gel matrix. ADH was further purified by FPLC Superdex 75 (Pharmacia) in the same buffer at 0.5 ml/min. Fractions were tested by SDS-electrophoresis and protein was quantified by the method of Bradford [19].

2.3. Recombinant ADH analysis

SDS-PAGE was performed in 12.5% acrylamide gels with Coomassie-blue staining. Western blotting was performed [20] using a monoclonal antibody against *Drosophila* ADH (LLBE8). ADH activity was measured spectrophotometrically by the increase in absorbance at 340 nm, using 100 mM ethanol, 100 mM 1-propanol, 100 mM 2-propanol, 100 mM 1-butanol and 50 mM 2-butanol and 1.8 mM NAD^+. A range of pH's from 7.0 to 9.0 was assayed using 2-propanol as substrate.

2.4. Structural analysis of the catalytic triad

D. lebanonensis active site coordinates were from X-ray analysis data [3] and a refined crystallographic model ($R = 20.1\%$, $R_{free} = 24.0\%$, resolution range = 8–1.92 Å). Crystal structures were from the Brookhaven Protein Data Bank (PDB): 3α,20β-HSDH (PDB code: 2HSD) [11,12], DHPR (PDB code: 1DHR) [15,21] and UDP (PDB code: 1UDP) [22]. Only Cα coordinates were available for UDP, so the program O [23] was used to build the main chain from Cα positions. Side-chains were chosen from the rotamer library facility of the same program and according to published data on the active site of UDP [22]. Comparison of the triad Ser-Tyr-Lys was performed by least squares superposition of 3α,20β-HSDH, DHPR and UDP Cα positions on DADH using the O program. The positions analyzed were: 138–159 of DADH, 138–159 of 3α,20β-HSDH and 132–153 of DHPR. Due to an insertion, two segments of UDP (123–129 and 147–156) were compared to DADH. Fig. 1 was produced in MOLSCRIPT [24] and rendered by RASTER3D [25,26].

3. Results

3.1. Mutagenesis: purification and characterization of the mutant enzymes

Landt's PCR procedure used to construct the $Ser^{139}Cys$ gene gave a unique product of the expected size (770 bp) but repeatedly failed to amplify a band of the correct size in the second step of the $Ser^{139}Ala$ mutagenic PCR. Thus, the $Ser^{139}Ala$ gene was constructed following Picard [18]. Sequencing of the mutant genes cloned in pKK223-3 revealed that the mutagenized codon was present and that no other unwanted substitutions had been introduced. SDS-PAGE of total protein extracts obtained from small scale cultures of the *E. coli* cells transformed with the plasmids containing the wild-type and the two mutants confirmed the synthesis of the three recombinant enzymes in similar yield. Recombinant *D. melanogaster* ADH did not bind to Blue Sepharose as other DADHs [27,28], but was purified in a 1 ml fraction of a Superdex 75 step in an alternative protocol [28]. The only band detectable by SDS-PAGE was recognized in Western blot analysis by the anti-*D. melanogaster*-ADH mAb LLBE8.

Concentrations of ADH in the purified preparations were 0.235 μg/μl for the wild-type enzyme, 0.167 μg/μl for the $Ser^{139}Cys$ mutant and 0.135 μg/μl for the $Ser^{139}Ala$ mutant. Enzymatic assays performed under standard conditions (1 min at 25°C, using 2-propanol as substrate at pH 8.6) showed that while the wild-type form was fully active (0.801 mU/μl), neither mutant showed detectable activity. Activity was also checked with ethanol, 1-propanol, 1-butanol and 2-butanol at standard pH (8.6), and also at different pH's (7.0, 7.5, 8.0 and 9.0) with 2-propanol. No detectable activity was re-

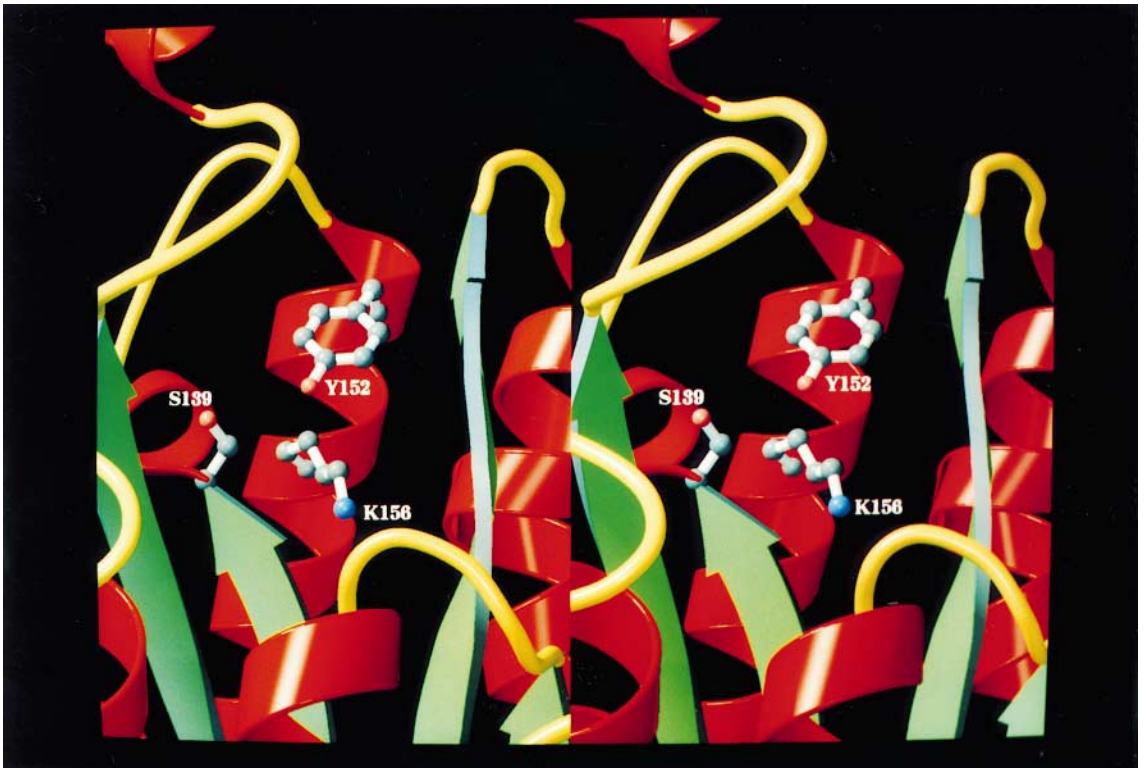

Fig. 1. Stereoview of the Ser^{139}-Tyr^{152}-Lys^{156} triad in the DADH active site based on a refined crystallographic structure, shown in ball-and-stick representation. Secondary structure elements are represented as red ribbons for α-helices, green arrows for β-strands and yellow coils for loops and turns, generated in MOLSCRIPT [24] and rendered by RASTER3D [25,26].

corded in any case, indicating that neither Ser^{139}Cys nor Ser^{139}Ala could oxidize any of the substrates.

3.2. *Relative position of Ser^{139} with respect to Tyr^{152} and Lys^{156} in DADH*

The conformation of the 138–158 protein segment was depicted based on a refined crystallographic model (Fig. 1). The spatial arrangement of Ser^{139} side-chain, facing Tyr^{152} and Lys^{156} fully confirms and extends the data obtained from site-directed mutagenesis. Least squares superposition of Cα atoms of DADH with the corresponding regions of available crystal structures of 3α,20β-HSDH, DHPR and UDP resulted in a root mean square deviation of: 1.1 Å for 3α,20β-HSDH, 1.2 Å for 6,7-DHPR and 1.0 Å for UDP, showing that the active site geometries in those SDR members diverge quite a bit from DADH.

4. Discussion

Suitable candidates for catalytic residues are those strictly conserved among the 57 reported DADH. Of those, Tyr^{152} and Lys^{156} had been previously confirmed by chemical modification and site-directed mutagenesis. Further involvement of Ser^{139} in DADH, although supported by crystallographic data on four SDR enzymes, 3α,20β-HSDH [11,12], 17β-HSDH [13], MLCR [14] and DHPR [15], required experimental confirmation. The null activity of the mutant forms now reported validates the contribution of Ser^{139} to catalysis and highlights the need of a side-chain hydroxyl group, whose absence or replacement by a thiol leads to a dead enzyme. In agreement with our data a Ser^{138}Ala replacement of human 15-hydroxyprostaglandin dehydrogenase, another SDR enzyme, produced crude extracts completely devoid of enzymatic activity [29], and substitution of Ser^{138} by Thr in 3β,17β-HSDH yielded an active protein with identical catalytic constants to the wild-type [16].

The structural information of the 138–158 fragment reported in this work was obtained from the refined X ray structure of DADH [3] and Benach et al. (to be published). Ser^{139} is located at the end of the strand βE, enclosed in the turn connecting βE and helix αF, Tyr^{152} is at the N-terminal end of the αF-helix and Lys^{156} is in the same α-helix (Fig. 1). The spatial configuration of the side chains of these three residues, all facing the inner side of the putative substrate binding pocket, supports their contribution to the reaction mechanism in full agreement with the catalytic triad of the 'SDR' family [2]. Our data on the Ser^{139} site-directed mutants, together with the previously reported chemical modification experiments [6,7] and amino acid substitutions on the Tyr^{152} and Lys^{156} [8,9] highlight its direct contribution to catalysis.

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