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Substitutions of a buried glutamate residue hinder the conformational change in horse liver alcohol dehydrogenase and yield a surprising complex with endogenous 3′-Dephosphocoenzyme A☆

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ABSTRACT

Glu-267 is highly conserved in alcohol dehydrogenases and buried as a negatively-charged residue in a loop of the NAD coenzyme binding domain. Glu-267 might have a structural role and contribute to a rate-promoting vibration that facilitates catalysis. Substitutions of Glu-267 with histidine or asparagine residues increase the dissociation constants for the coenzymes (NAD^+ by ~40-fold, NADH by ~200-fold) and significantly decrease catalytic efficiencies by 16–1200-fold various substrates and substituted enzymes. The turnover numbers modestly change with the substitutions, but hydride transfer is at least partially rate-limiting for turnover for alcohol oxidation. X-ray structures of the E267H and E267 N enzymes are similar to the apoenzyme (open) conformation of the wild-type enzyme, and the substitutions are accommodated by local changes in the structure. Surprisingly, the E267H and E267 N enzymes have endogenous (from the expression in *E. coli*) 3′-dephosphocoenzyme A bound in the active site with the ADP moiety in the NAD binding site and the pantethiene sulfhydryl bound to the catalytic zinc. The kinetics and crystallography show that the substitutions of Glu-267 hinder the conformational change, which occurs when wild-type enzyme binds coenzymes, and affect productive binding of substrates.

1. Introduction

Horse liver alcohol dehydrogenase (ADH1E) is a dimer with a molecular mass of 80 kDa with two identical subunits, each having a coenzyme binding domain with the Rossmann fold and a catalytic domain with the zinc ion to which substrates bind in the cleft between the domains [1,2]. Three-dimensional structures of the apoenzyme and a ternary complex with coenzyme and a substrate analogue have two different conformational states [3]. A rotation of 10° brings the catalytic domain with residues involved in activity closer to the coenzyme domain [4]. The conformational change of the enzyme-NAD^+ complex is controlled by deprotonation of the water bound to the catalytic zinc and precedes substrate binding [5]. The enzyme-NADH complex also isomerizes, but is too fast to determine with stopped-flow kinetics [6,7]. The minimal ordered bi bi mechanism is shown in Scheme 1 where step 2 represents the isomerization.

Several studies have shown that substitutions of amino acid residues in the active site decrease affinity for coenzymes and alter the conformational change [8]. Inspection of the ADH structure led us to examine the role of Glu-267, which is buried in a loop of the coenzyme binding domain connecting the end of a β-strand to an α-helix and includes Val-268 and Ile-269, whose carbonyl groups provide hydrogen bonds to the coenzyme [9]. The intrinsic p*K* of the γ-carboxyl group is 4.5 [10], and the hydrogen bonds (Fig. 1) would decrease the pK and stabilize a negatively-charged carboxylate at pH 7 [11].

Glu-267 is highly conserved in dimeric, vertebrate and plant ADHs, but the corresponding residue in other, especially tetrameric, ADHs can be Val, Asp, Asn, Leu or Met, and *Saccharomyces cerevisiae* ADH1 has Asn-244 [12–14]. How the various residues that occupy this position in ADHs affect structure and activity have not been studied.

Abbreviations: ADH, alcohol dehydrogenase; E267H, substitution Glu-267 with histidine residue; E267 H/N, substitution with either histidine or asparagine; MPD, 2-methyl-2,4-pentanediol; RMSD, root-mean-square deviation

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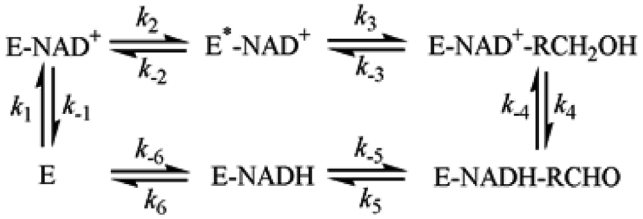

Scheme 1. Alcohol dehydrogenase kinetic mechanism.

Buried carboxylates can be important for catalysis. Asp-49 in horse ADH1E forms hydrogen bonds with the imidazole group of His-67 (which is ligated to the catalytic zinc) and to Gly-66 N, and via a buried water to Gly-44 O, His-67 N and Glu-68 N. When the homologous residue in yeast ADH, Asp-46, is changed to Asn, the catalytic activity decreases by 100–1000 fold [12,15]. This aspartic acid residue is highly conserved in ADHs and may modulate the electrostatic potential of the zinc.

The contribution of Glu-267 is also of interest because it was suggested to be one of eight residues in a conserved evolutionary sequence (residues 144–292) that participates in a rate-promoting vibration that is coupled to the hydride transfer catalyzed by horse liver ADH [16–18]. Fast protein motions can compress the active site and lower the barrier to reaction because hydrogen tunneling is very dependent on the donor-acceptor (alcohol-NAD^+) distance [19–23]. Computational studies with yeast alcohol dehydrogenase and lactate dehydrogenase support roles for rate-promoting vibrations in hydride transfers [24–27].

Because substitutions of buried, charged amino acid residues can alter protein structure and stability [11,28,29], we made conservative, almost isosteric substitutions of Glu-267 with the neutral Gln, Asn, and His residues. The Gln-267 enzyme was poorly expressed, and even the small structural changes with the Asn-267 and His-267 enzymes significantly affected enzymatic activity, coenzyme binding and the conformational change. The conformations of the substituted proteins were most similar to the "open" ADH apoenzyme. Surprisingly, the active sites were occupied by endogenous 3′-dephosphocoenzyme A, as isolated from the expression system in *E. coli*. The binding of endogenous ligands from the cytosol may be unusual, but a beautiful example is the blue fluorescent protein from the blue walleye where a lipocalin was discovered to bind biliverdin, a degradation product of heme [30].

2. Materials and methods

2.1. Reagents

NAD^+ (grade I, Li salt) and NADH (grade I, disodium salt) were purchased from Roche Boehringer Mannheim; deuterated alcohols were from MSD Isotopes; DEAE-Sepharose Fast Flow and SP-Sepharose Fast Flow were from Amersham Biosciences; Quick Change Site-Directed mutagenesis kit was from Stratagene; QIAprep Spin Miniprep Kit came from Qiagen; oligonucleotide primers were synthesized at Integrated DNA Technologies.

2.2. Site-directed mutagenesis

The expression plasmid pBPP/*Eq*ADH [31] containing the cDNA for horse liver ADH1E (*Equus caballus*, EC 1.1.1.1, NCBI taxonomy ID 9796, UniProt P00327, GenBank M64864) was used to produce the substitutions of Glu-267 using the Quick Change method and partially random mutagenesis with the oligodeoxyribonucleotide 5′-GGT GTG GAT TTT TCC TTT (A/C)A(A/C) GTC ATT GGT CGG CTC GAC-3′ as one primer and its complimentary sequence as the other primer, where the underline marks the site of mutation. The mutagenesis provided two plasmid preparations for each of the substitutions of the codon for Glu (GAA) with those for Asn (AAC), Gln (CAA) and His (CAC), as determined by sequencing in the University of Iowa DNA Facility with a primer with the sequence 5′-GGATGGTACCAGCAGGTT-3'. *Escherichia coli* strain XL1-Blue cells (Stratagene) were transformed and grown in LB medium containing 100 μg/ml ampicillin and 12 μg/ml tetracycline with induction by 0.5 mM isopropyl β-D-1-thiogalactopyranoside. Cells were lysed and the supernatant fraction was analyzed by gel electrophoresis on nondenaturing (native) agarose gels at pH 8 where ADH migrates toward the cathode, and almost all of the *E. coli* proteins migrate toward the anode [31]. The E267 N and E267H enzymes were active and expressed well as found by staining for enzyme activity and protein. However, the E267Q enzyme was poorly expressed and apparently inactive.

2.3. Purification and characterization of enzymes

The substituted ADHs were expressed and purified as previously described [31]. The buffers contained 0.25 mM EDTA, which protects the enzyme, and the steps were done at 4 °C. Cells from LB media were resuspended at 1 g wet cells per 3 ml of 10 mM Tris-HCl buffer, pH 8. The suspension was treated with egg white lysozyme (0.25 mg/ml) for 30 min at 4 °C with stirring, followed by ultrasonification (15 alternating cycles: on for 30 s and off for 2 min). Protamine sulfate (2 mg/ml) was added, and the suspension was stirred for 1 h. The supernatant was collected by centrifugation for 50 min at 15,000 rpm and applied to a DEAE-Sepharose column equilibrated with 10 mM Tris-HCl buffer, pH 8. The enzyme passes through the column and was dialyzed against 5 mM sodium phosphate buffer, pH 7.0. The solution was applied to an SP-Sepharose column developed with a gradient from 5 to 100 mM sodium phosphate buffer, pH 7.0. The amount of "contaminating" adenosine nucleotide was decreased by passing the concentrated enzyme through a second DEAE-Sepharose column equilibrated with 10 mM Tris-HCl buffer, pH 7.5. The enzymes were homogeneous as judged by SDS-PAGE.

Protein concentrations were determined by UV absorbance with correction for variable concentrations of adenosine nucleotide, using extinction coefficients of $\varepsilon_{280} = 18{,}100\ M^{-1}cm^{-1}$, $\varepsilon_{260} = 12{,}300\ M^{-1}cm^{-1}$ for the 40 kDa protein subunit and of $\varepsilon_{280} = 3900\ M^{-1}cm^{-1}$ and $\varepsilon_{260} = 14{,}000\ M^{-1}cm^{-1}$ applicable for enzyme-bound adenosine 5′-diphosphoribose [32]. Two equations with the two concentrations to be determined were solved with the A_{260} and A_{280} observed from a UV spectrum. Because the substituted enzymes were somewhat unstable and the binding of NAD^+ and pyrazole was weak, the concentrations of active sites were not determined accurately by titration with NAD^+ in the presence of pyrazole, even though some change in absorbance at 292 nm was observed [33]. A standard activity assay was used to determine enzyme concentrations for calculating turnover numbers from various kinetic studies [34].

2.4. Steady-state kinetics

The concentrations of the coenzymes NAD^+ and NADH were determined by absorbance at 260 nm and 340 nm, respectively. Concentrations of benzyl alcohol and benzaldehyde were determined by absorbance, $\varepsilon_{257} = 200\ M^{-1}cm^{-1}$ and $\varepsilon_{249} = 12{,}500\ M^{-1}cm^{-1}$, respectively. All kinetic studies used 33 mM sodium phosphate, 0.25 mM EDTA, buffer at pH 8.0 and 25 °C. Initial velocities were determined on a Cary 118C spectrophotometer with computer fitting of the progress curves to a linear or parabolic function. Initial velocity studies for the forward and reverse reactions used a systematically varied 5X5 matrix of concentrations of both substrates and coenzymes (duplicate assays), and data were fitted to the equation for a sequential bi mechanism, $v = VAB/(1 + K_aB + K_bA + K_{ia}K_b)$, in order to obtain true K_m and V_{max} values [35]. Product inhibition was also used to obtain the inhibition (dissociation) constants for coenzymes, and data were fitted to the equation for competitive inhibition. Dead-end