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Supporting role of lysine 13 and glutamate 16 in the acid–base mechanism of saccharopine dehydrogenase from *Saccharomyces cerevisiae*

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ABSTRACT

Saccharopine dehydrogenase (SDH) catalyzes the NAD^+ dependent oxidative deamination of saccharopine to form lysine (Lys) and α-ketoglutarate (α-kg). The active site of SDH has a number of conserved residues that are believed important to the overall reaction. Lysine 13, positioned near the active site base (K77), forms a hydrogen bond to E78 neutralizing it, and contributing to setting the pK_a of the catalytic residues to near neutral pH. Glutamate 16 is within hydrogen bond distance to the Nε atom of R18, which has strong H-bonding interactions with the α-carboxylate and α-oxo groups of α-kg. Mutation of K13 to M and E16 to Q decreased k_{cat} by about 15-fold, and primary and solvent deuterium kinetic isotope effects measured with the mutant enzymes indicate hydride transfer is rate limiting for the overall reaction. The pH-rate profiles for K13M exhibited no pH dependence, consistent with an increase in negative charge in the active site resulting in the perturbation in the pK_as of catalytic groups. Elimination of E16 affects optimal positioning of R18, which is involved in binding and holding α-kg in the correct conformation for optimum catalysis. In agreement, a $\Delta\Delta G^{\circ\prime}$ of 2.60 kcal/mol is estimated from the change in $K_{\alpha\text{-kg}}$ for replacing E16 with Q.

Introduction

Saccharopine dehydrogenase (N6-(glutaryl-2)-L-lysine: NAD^+ oxidoreductase; EC 1.5.1.7) (SDH)[1] catalyzes the reversible pyridine nucleotide dependent oxidative deamination of saccharopine (Sacc) to generate α-ketoglutarate (α-kg) and lysine (Lys) using NAD^+ as an oxidant [1,2]. An acid–base chemical mechanism has been proposed for the *Saccharomyces cerevisiae* enzyme (*Sc*SDH), Scheme 1 [3]. Recent site-directed mutagenesis studies are consistent with K77 and H96 as acid–base catalysts in the reaction [4]. In the direction of Sacc formation, once the E·NADH·α-kg·Lys central complex is formed, the ε-amine of K77 accepts a proton from the ε-amine of Lys prior to attack of the imine on the α-carbonyl group of α-kg (I). The resulting carbinolamine is protonated by H96 (II). The conjugate base of H96 then accepts a proton from the carbinolamine nitrogen, and donates it to the leaving hydroxyl to form water (III). The imine is then reduced by NADH concomitant with protonation of the imine nitrogen by the conjugate acid of K77 (IV) [4]. Isotope effects and pH-rate profiles for the K77M and H96Q mutant enzymes are consistent with the proposed roles of K77 and H96 [4]. A structure of the E·NADH·Sacc ternary complex is also consistent with the suggested roles of the two enzyme residues [4], Fig. 1.

In the SDH active site, there are a number of ionizable residues including D319, E16, E78, E122, H96, K13, K77, K99, R18 and R131 (Fig. 1) [5,6]. An alignment of the primary sequences of SDH from *Candida albicans*, *Pichia guilliermondii*, *S. cerevisiae*, *Aspergillus fumigatus*, and *Cryptococcus neoformans* indicated all of the ionizable residues are conserved (data not shown), suggesting they are all important to the overall reaction.

Two cysteine residues, C205 and C249, in the dinucleotide binding site form a disulfide bond as observed in the crystal structure of the WT SDH apo-enzyme [5]. In the AMP-bound structure, these residues are in the reduced, dithiol, form [5]. A mutation of C205 to S, gave a mutant enzyme with much higher rates at pH 7 and below compared to the WT enzyme, but with essentially identical kinetic and chemical mechanisms [7]. The C205S mutant enzyme is thus used as a pseudo-WT enzyme and is the frame of reference for site-directed mutagenesis studies with mutations generated in a C205S background [4,8].

According to the crystal structure of the E·NADH·Sacc tertiary complex [4], one of the conserved residues, K13, is within hydrogen bond distance to E78 and D319. Another conserved residue, E16, is within hydrogen bond distance to R18 and has strong

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[1] *Abbreviations used*: AAA, α-aminoadipate pathway; SDH, saccharopine dehydrogenase; α-kg, α-ketoglutarate; Sacc, L-saccharopine; Lys, L-lysine; NAD^+, β-nicotinamide adenine dinucleotide; NADH, reduced β-nicotinamide adenine dinucleotide; NADD, reduced β-nicotinamide adenine dinucleotide with deuterium in the 4-R position; WT, wild type; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Taps, 3-[*N*-tris(hydroxymethyl) methylamino]-propanesulfonic acid; Hepes, *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid); Ches, 2-(*N*-cyclohexylamino) ethanesulfonic acid; PKIE, primary kinetic isotope effect; SKIE, solvent kinetic isotope effect; MKIE, multiple kinetic isotope effect.

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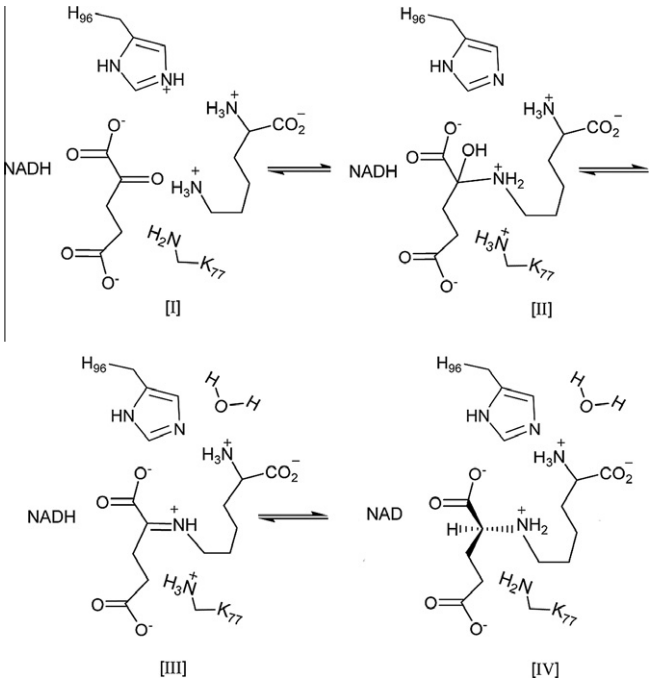

Scheme 1. Chemical mechanism proposed for saccharopine dehydrogenase. The reaction is written in the direction of Sacc formation. [I], the formed central complex E·NADH·α-kg·Lys; [II], carbinolamine intermediate; [III], imine; [IV], the generated central complex E·NAD$^+$·Sacc. With the exception of Sacc, no stereochemistry is implied.

hydrogen-bonding interactions to the α-carboxyl- and α-oxo-groups of α-kg, Fig. 1 [4]. In this study the roles of K13 and E16 have been studied. The two residues were mutated to M and Q, respectively, which were expected to provide a significant change in active site charge and affect the pK_as of the catalytic residues. In addition, the two residues likely contribute to binding of α-kg and Lys. Mutant enzymes were characterized via the pH dependence of kinetic parameters and isotope effects. Data are discussed in terms of the proposed general acid–general base mechanism of SDH.

Materials and methods

Chemicals

Ampicillin, chloramphenicol, Sacc, Lys, α-kg, horse liver alcohol dehydrogenase, and yeast aldehyde dehydrogenase were obtained from Sigma. β-NADH, β-NAD$^+$, Luria–Bertani (LB) broth, LB-agar, and imidazole were purchased form US Biochemical Corp. The buffers, Ches, Taps, Hepes, and Mes, were from Research Organics, while Ni^{2+}–NTA resin was purchased from 5 Prime. Ethanol-d_6 (99 atom% D) and D_2O (99.9 atom% D) were purchased from Cambridge Isotope Laboratories. Ethanol (absolute, anhydrous) was from Pharmaco-Aaper. Isopropyl-β-D-1-thiogalactopyranoside was from Invitrogen, and the GenElute plasmid miniprep kit was from Sigma. The QuikChange site-directed mutagenesis kit was from Stratagene. 4*R*-4-^{2}H NADH was prepared as described previously [9]. The concentration of NADD was estimated using a ε_{340} of 6220 $M^{-1}cm^{-1}$. All chemicals were obtained commercially, were of the highest grade available and were used without further purification.

Site-directed mutagenesis

Template DNA used for site-directed mutagenesis was the plasmid containing SDH with the C205S mutation, [7], to change K13 and E16 to M and Q, respectively. The forward and reverse primers used to generate the K13M mutant enzyme are as follows: $K13_f$,5′-CTAAGAGCTGAAACT**ATG**CCCCTAGAGGCACGTG-3′; $K13_r$, 5′-CACGTGCCTCTAGGGG**CAT**AGTTTCAGCTCTTAG-3′. Primers used to generate the E16Q mutant enzyme are as follows: $E16_f$, 5′-GAAACTA AACCCCTA**CAG**GCACGTGCTGCC-3′; $E16_r$, 5′-GGCAGCACGTGC**CTG**T AGGGGTTTAGTTTC-3′. The mutated codons are shown in bold. Mutagenesis was carried out according to the instructions in the QuikChange site-directed mutagenesis kit as described previously [10]. The XL-1-Blue competent cell strain of *Escherichia coli* was transformed with the plasmids containing mutations. Plasmids were isolated and purified using the GenElute plasmid mini preparation kit. Mutations were confirmed by sequencing the entire gene at the Sequencing Core of the Oklahoma Medical Research Foundation, Oklahoma City, OK. As described above, the C205S mutant enzyme is pseudo-WT and double mutant enzymes K13M/C205S and E16Q/C205S will be referred to throughout as K13M and E16Q.

Expression and purification

Escherichia coli BL21 (DE3)-RIL cells were transformed with plasmids containing mutant genes and expression was carried out as reported previously [2] with some modifications. Once cell density reached an A_{600} of 0.5–0.6, induction of protein expression was carried out at 37 °C by addition of 0.1 mM IPTG, followed by a 16 h incubation at 25 °C. Cells were harvested by centrifugation at 10,000*g* for 10 min. Cells were then sonicated in 100 mM Hepes, pH 7.5, containing 300 mM NaCl, 5 mM imidazole. Enzymes were purified by Ni^{2+}–NTA affinity chromatography, with elution at

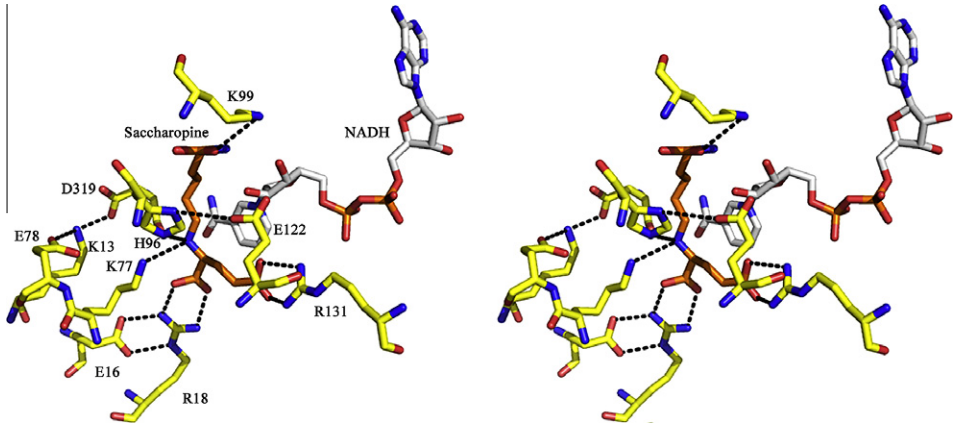

Fig. 1. Stereoview of the active site of SDH in the ternary complex with NADH and saccharopine (pdb 3UH1). Residues within hydrogen bond distance (shown as a dashed line) to NADH and Sacc are shown. Distances for the hydrogen bonds range from 2.6 to 2.8 Å.