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Contribution of K99 and D319 to Substrate Binding and Catalysis in the Saccharopine Dehydrogenase Reaction

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Abstract

Saccharopine dehydrogenase catalyzes the NAD-dependent oxidative deamination of saccharopine to L-lysine and α -ketoglutarate. Lysine 99 is within hydrogen-bond distance to the α -carboxylate of the lysine substrate and D319 is in the vicinity of the carboxamide side chain of NADH. Both are conserved and may be important to the overall reaction. Replacing K99 with M gives decreases of 110-, 80- and 20-fold in the V_2/K_m values for lysine, α -ketoglutarate and NADH, respectively. Deuterium isotope effects on V and V/K_{Lys} increase, while the solvent deuterium isotope effects decrease compared to the C205S mutant enzyme. Data for K99M suggest a decreased affinity for all reactants and a change in the partition ratio of the imine intermediate to favor hydrolysis. A change in the bound conformation of the imine and/or the distance of the imine carbon to C4 of the nicotinamide ring of NADH is also suggested. Changing D319 to A decreases V_2/K_{NADH} by 33-fold. Primary deuterium and solvent deuterium isotope effects decrease compared to C205S suggesting a non-isotope sensitive step has become slower. NADH binds to enzyme first, and sets the site for binding of lysine and α -ketoglutarate. The slower step is likely the conformational change generated upon binding of NADH.

1 Introduction

Saccharopine dehydrogenase catalyzes the final step of α -amino adipate (AAA1) pathway for lysine biosynthesis, a pathway unique to fungi and euglenoids [1–3]. While lysine is an essential amino acid for mammals, human pathogenic fungi, including *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* and the plant pathogen *Magnaporthe grisea*, use the AAA pathway for lysine biosynthesis [1, 2, 4]. Knocking out the *LYS1* gene, which encodes SDH is lethal to fungal cells, suggesting that selective inhibition of one or more enzymes in the pathway may help to control or completely eradicate these pathogens *in vivo* [4, 5]. Therefore, the enzymes of this pathway are potential targets for designing effective antimycotic drugs.

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¹Abbreviations: SDH, saccharopine dehydrogenase; AAA, α -amino adipate; Lys, lysine; α -Kg, α -ketoglutarate; Sacc, saccharopine; NAD, nicotinamide adenine dinucleotide [the + charge is omitted for convenience]; OG, oxalylglycine; NADH, reduced nicotinamide adenine dinucleotide; NADD, reduced nicotinamide adenine dinucleotide with deuterium in the 4R position; WT, wild type; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid, Ches, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; Taps, [3-*N*-tris(hydroxymethyl)methyl]-3-amino-propanesulfonic acid; D₂O, deuterium oxide; DCl, deuterium chloride; NaOD, sodium deuterioxide; LB, Luria-Bertani; IPTG, isopropyl β -D-1-thiogalactopyranoside; Ni-NTA, nickel-nitrilotriacetate; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Saccharopine dehydrogenase [SDH; N6-(glutaryl-2)-L-lysine: nicotinamide adenine dinucleotide (NAD) oxidoreductase (L-lysine forming); (EC 1.5.1.7)] catalyses the reversible pyridine nucleotide-dependent oxidative deamination of saccharopine (Sacc) using NAD as the oxidizing agent to produce lysine and α -ketoglutarate (α -Kg), Scheme 1 [6]. The dehydrogenase from *S. cerevisiae* is a monomer with a molecular weight of about 41,000, with one active site [7].

Structures of SDH have been solved in the apo-enzyme form [8] and with either AMP or oxalylglycine (OG) bound, analogues of NAD and α -Kg, respectively [9]. In structures of the apoenzyme, a disulfide bond between C205 and C249 (in *S. cerevisiae*) is observed in close proximity to the dinucleotide-binding site [8]. However, in a structure solved with AMP bound to the dinucleotide site the disulfide is reduced (S-S distance: 3.64Å) [6]. Data suggested the binding of the dinucleotide substrates was hampered in the oxidized enzyme, and this was confirmed upon characterization of the C205S mutant enzyme [11]. The activity of the recombinant enzyme, as isolated, was somewhat variable depending on the oxidation state of the disulfide [11]. The C205S mutant enzyme was prepared, and found to have stable activity, with a kinetic and chemical mechanism qualitatively identical to that of the native enzyme. The C205S mutant SDH is the reference, or pseudo-WT enzyme for all site-directed mutagenesis studies, and all mutant enzymes are prepared in the C205S background.

A semi-empirical ternary complex structure of the E-NAD-Sacc ternary complex was generated on the basis of the superposition of the E-AMP and E-OG structures and building in the NMN moiety of NAD and the lysine moiety of Sacc, Figure 1 [9]. Given the semi-empirical nature of the model, the relative positions of reactants and active site groups are estimates, and the overall model represents an open form of the enzyme, e.g., the distance for hydride transfer from the Ca proton of the glutamyl moiety of Sacc to the 4 position of the nicotinamide ring is 4.7 Å, much longer than van der Waals contact distance. There are a number of ionizable residues in the active site, and a multiple sequence alignment (data not shown) of the SDH from *Saccharomyces cerevisiae*, *Candida albicans*, *Pichia guilliermondii*, *Aspergillus fumigatus*, *Cryptococcus neoformans*, and *Ashbya* sp. indicated all are conserved in these six organisms, consistent with their importance in the mechanism. In the ternary complex, R131 and R18 are likely ion-paired to two of the carboxylates of Sacc. In addition, there are three lysine residues, K99 in the vicinity of the α -carboxylate of Sacc, K77 in the vicinity of the secondary amine of Sacc, and K13 near K77; three glutamate residues, E122 near K99, E78 near K77 and K13, and E16 near R18; and an imidazole, H96. In the ternary complex, the nicotinamide ring of NAD is positively charged, but in the vicinity of D319, and the secondary amine of Sacc is positively charged given its pK_a of about 10 [10]. The active site is positively charged, and this will certainly affect the pK_a values of all of the ionizable residues in the site.

In this manuscript the roles of the conserved residues, K99 and D319, Figure 2, were studied in the background of the C205S mutant enzyme, by changing them to methionine, which has the same side chain volume as lysine, and alanine, which eliminates the carboxylate functional group, respectively. On the basis of the proposed semi-empirical model, eliminating the charges on these residues is expected to disturb the fine balance of the hydrogen bonding network in the site, disrupting the integrity of the active site, and giving effects on the binding of substrates, and the pK_a values of other active site residues, including the catalytic groups. Mutant enzymes were prepared in the C205S background, which eliminates disulfide formation, so that 100% of the enzyme is in the “reduced” active form [11]. The K99M/C205S and D319A/C205S mutant enzymes were completely characterized via the pH dependence of kinetic parameters and isotope effects. Data are discussed in terms of the proposed mechanism of SDH.

2 Materials and Methods

2.1 Materials

Imidazole, Ches, Hepes, Mes, Taps, and Tris were from Research Organics. β -NADH, β -NAD, Luria–Bertani (LB) broth, LB-agar and imidazole were purchased from USB. L-L-Saccharopine, L-lysine, α -ketoglutarate, ampicillin, chloroamphenicol, baker's yeast alcohol and aldehyde dehydrogenases, and the Gen-elute plasmid miniprep kit were obtained from Sigma. Ethanol- d_6 (99% atom D) and D_2O (99.9% atom D) were purchased from Cambridge Isotope Laboratories. Ethyl alcohol (absolute, anhydrous) was from Pharmaco-Aaper. Isopropyl- β -D-1-thiogalactopyranoside (IPTG) was from Invitrogen. Oxalylglycine was from Echelon. Ni-NTA agarose resin was from Qiagen. The QuikChange site-directed mutagenesis kit was from Stratagene. All chemicals were obtained commercially, were of the highest grade available, and were used without further purification.

4-R-4- 2H NADH and NADH were prepared as previously described [12]. The purity of the final NADH(D) was estimated by measuring the absorbance ratio at 260/340 nm; a ratio of 2.27 ± 0.06 was obtained similar to the value of 2.15 ± 0.05 expected for pure compound [12]. The concentration of NADH(D) was estimated using a ϵ_{340} of $6220 \text{ M}^{-1}\text{cm}^{-1}$. The initial rates were similar when measured using the same concentrations of commercial NADH and the NADH prepared as above. The NADH(D) was used immediately after preparation without further purification.

2.2 Site-directed mutagenesis

Site-directed mutagenesis was performed using the plasmid carrying the C205S mutation of the *LYSI* gene [11] from *S. cerevisiae* in order to change K99 and D319 to M and A, respectively. The C205S mutant enzyme is used as the background in place of wild type, since it cannot form a disulfide linkage with the nearby C249 [11]. The disulfide form of the enzyme has much lower values of V and V/K_{NAD} at low pH. The forward and reverse primers used to generate the C205S/K99M and C205S/D319A mutated genes are listed in the Table 1. The entire gene insert was then sequenced at the Laboratory for Gene Sequencing of the Oklahoma Medical Research Foundation (OMRF) in Oklahoma City, OK. The double mutant enzymes obtained, C205S/K99M and C205S/D319A, will be referred to as K99M and D319A, with C205S as reference and referred to as pseudo-wild type.

2.3 Expression and purification of mutant enzymes

E. coli BL21 (DE3) RIL cells were transformed with plasmids containing the K99M and D319A mutant genes. All mutant proteins were expressed as previously described [6], using IPTG for induction. Enzymes were purified using Ni-NTA affinity chromatography. Proteins bound to the Ni-NTA column eluted at 300 mM imidazole at pH 8. Proteins were dialyzed against 100 mM Tris-HCl, 300 mM KCl at pH 8 and 4 °C, for 2–6 hours. Purity of the proteins was assessed using SDS–PAGE as previously described [6], and the protein concentration was measured using the Bradford reagent, measuring the absorbance at 595 nm with BSA as the standard [14].

2.4 Enzyme assay

The SDH reaction was monitored via the disappearance of NADH at 340 nm ($\epsilon_{340} = 6220 \text{ M}^{-1}\text{cm}^{-1}$) or at 366 nm ($\epsilon_{366} = 3110 \text{ M}^{-1}\text{cm}^{-1}$), using a Beckman DU 640 spectrophotometer. Reactions were initiated by the addition of enzyme to a mixture containing all other reaction components, and the initial linear portion of the time course was used to calculate the initial velocity. The amount of enzyme added was determined using an enzyme concentration series (v versus $[E]$) for each mutant enzyme.

Initial velocity patterns were obtained for the K99M and D319A mutant enzymes in the direction of Sacc formation. All data were collected at 25°C in 100 mM K⁺-Hepes, pH 7.2. Initial velocities were measured as a function of Lys concentration (0.5–10 K_m) at different fixed levels of α -Kg (0.5–10 K_m) with NADH maintained near saturation ($\geq 7.5 K_m$). Initial velocity studies were also carried out varying Lys concentration (0.5–10 K_m) at different fixed levels of NADH (0.5–10 K_m) with α -Kg maintained near saturation ($\geq 10 K_m$). No effect of NaCl/KCl up to 1.3 M was observed.

Inhibition patterns were measured for the D319A mutant enzyme at pH 6 using oxalylglycine (OG), a structural analog of α -Kg, as a dead-end inhibitor. NADH was varied at different fixed concentrations of OG (0, 3, 6, and 9 mM) with Lys and α -Kg maintained at low concentration (1.5 K_m). An app K_i for OG was first determined via Dixon plot ($1/v$ versus [OG]), with the rate measured as a function of OG, while maintaining all three substrates at 1.5 K_m .

2.5 pH studies

In order to determine whether the mutations affected the pK_a values observed in the pH-rate profiles of the wild type SDH, the initial velocity was measured in the direction of Sacc formation as a function of pH at 25°C. For the D319A mutant enzyme, the pH dependence of V and the V/K was measured by varying Lys as a function of pH (5–10), maintaining α -Kg at saturation (10 $K_{\alpha\text{-Kg}}$) and NADH at near saturation (5 K_{NADH}). The experiment was repeated for NADH, at fixed saturating concentrations of Lys and α -Kg (10 K_m). However, for K99M, the app K_m values obtained for Lys and NADH, at pH 6 and 9 were high, saturating levels could not be achieved for the two substrates; therefore, activities were measured over the pH range of 6 to 9. The pH was maintained using the following buffers at 100 mM concentration: K⁺-Mes 5.3–6.8; K⁺-Hepes, 6.8–8.2; K⁺-Ches; 8.2–10.3. Sufficient overlap was obtained upon changing buffers to observe potential buffer effects, and none were observed. The pH was recorded immediately after measuring the initial velocity at 25°C.

2.6 Isotope effects

Isotope effects were measured for the D319A mutant enzyme, in the pH independent region(s) of its pH-rate profile, pH 5.5 and 9. For K99M, substrate deuterium kinetic isotope effects were measured only at pH 7. Effects were measured in the direction of Sacc formation, using NADD as the deuterated substrate [6]. $^D V_2$ and $^D(V_2/K_{\text{Lys}})$ were obtained for both mutant proteins, by measuring initial rates in triplicate, as a function of Lys concentration (0.5–5 K_m), at saturating levels of α -Kg (10 K_m) and NADH(D) (7.5 K_m). The highest concentration of NADH(D) was 1.48 mM, which would give an absorbance >9 at 340 nm with a 1 cm pathlength. At the high concentrations, 0.4 cm pathlength cuvettes were used at a wavelength of 366 nm, where the extinction coefficient for NADH(D) is 3.11 $\text{mM}^{-1}\text{cm}^{-1}$.

Solvent deuterium kinetic isotope effects were obtained by direct comparison of initial rates, in triplicate, in H₂O and D₂O, at pH(D) 7 for K99M and D319A in the pH(D) independent region of the pH-rate profiles. Initial rates were measured varying Lys at fixed saturating levels of NADH (7.5 K_{NADH}) and α -Kg ($\geq 10K_m$). Reactions were initiated by adding a small amount of each of the mutant enzymes in H₂O to the reaction mixtures in H₂O or D₂O. For rates measured in D₂O, substrates (NADH, α -Kg, and Lys) and buffers were first dissolved in a small amount of D₂O and lyophilized overnight to remove exchangeable protons. The lyophilized powders were then dissolved in D₂O to give the desired concentrations, and the pD was adjusted using either DCl or NaOD. A value of 0.4 was added to pH meter readings to calculate pD [15].

Multiple isotope effects were determined in the direction of Sacc formation by direct comparison of the initial rates in H₂O and D₂O as above, at pH(D) 7, varying Lys at a fixed saturating concentration of NADD ($7.5K_{NADD}$) and α -Kg ($\geq 10K_m$).

2.7 Data analysis

Initial rate data were first analyzed graphically using double-reciprocal plots of initial velocity *versus* substrate concentration. Double reciprocal plots, suitable secondary and tertiary plots were visually evaluated to determine the quality of the data and the proper rate equations for data fitting. Data were fitted using the appropriate rate equations and programs developed by Cleland [16] or the Marquardt-Levenberg algorithm [17], supplied with the Enzfitter program from BIOSOFT, Cambridge, U.K. Kinetic parameters and their corresponding standard errors were estimated using a simple weighting method. Data obtained from the initial velocity patterns, in the absence of added products, were fitted using either eq.1 for a sequential mechanism, eq. 2 with the constant term absent, or eq. 3 for competitive inhibition by B, in a sequential mechanism. Data obtained from dead-end inhibition studies were fitted using eq 4, for uncompetitive inhibition.

$$v = \frac{V_{AB}}{K_{ia}K_b + K_aB + K_bA + AB} \quad (1)$$

$$v = \frac{V_{AB}}{K_aB + K_bA + AB} \quad (2)$$

$$v = \frac{V_{AB}}{(K_{ia}K_b + K_aB) \left(1 + \frac{B}{K_{IB}}\right) + K_bA + AB} \quad (3)$$

$$v = \frac{VA}{K_a + A(1 + I/K_{ii})} \quad (4)$$

In equations 1–4, v and V are initial and maximum velocities, **A**, **B** and **I** are substrate and inhibitor concentrations, and K_a and K_b are Michaelis constants for substrates A and B, respectively. In eqs. 1 and 3, K_{ia} is the dissociation constant for A from the EA complex and K_{IB} is the substrate inhibition constant for B. In eq. 4, K_{ii} is the intercept inhibition constant.

Data for pH-rate profiles that decreased with a slope of 1 at low pH and a slope of -1 at high pH were fitted using eq 5. Data for the D319A V_2/E_t pH-rate profile were fitted to eq. 6.

$$\log y = \log \left[C / \left(1 + \frac{H}{K_1} + \frac{K_2}{H} \right) \right] \quad (5)$$

$$\log y = \log \left[\frac{Y_L + Y_H (K_1/H)}{1 + (K_1/H)} \right] \quad (6)$$

In eqs. 5–6, y is the observed value of the parameter (V or V/K) at any pH, C is the pH independent value of y , H is hydrogen ion concentration, K_1 , and K_2 represent acid dissociation constants for enzyme or substrate functional groups, Y_L and Y_H are constant values of V at low and high pH, respectively.

Isotope effect data were fitted using eq. 7, which allows the isotope effects on V and V/K to be independent of one another.

$$v = \frac{VA}{K_a(1+F_iE_{V/K})+A(1+F_iE_V)} \quad (7)$$

In eq. 7, F_i is the fraction of deuterium label in the substrate or D_2O , $E_{V/K}$ and E_V are the isotope effects minus 1 on V/K and V , respectively. All other parameters are as defined above.

3 Results

3.1 Cell growth, protein expression, and purification

Expression of the K99M and D319A mutant enzymes was nearly two times lower than that of C205S using the same growth conditions. All mutant proteins were eluted from the Ni-NTA column with buffer containing 300 mM imidazole at pH 8. The final enzyme preparations were judged to be >98% pure. The amount of purified enzyme obtained from a 1 L culture for the K99M and D319A mutant proteins was 2.5 and 2.0 mg, respectively. His-tagged mutant proteins were active and stable for 5–6 months when kept at 4°C in 100 mM Tris-HCl and 300 mM KCl at pH 8. Enzymes are active as shown below, suggesting that changes in structure, if any, are localized to the reactant binding sites.

3.2 Initial velocity studies of the K99M and D319A mutant enzymes

Double reciprocal initial velocity patterns were obtained at pH 7.2 in the direction of Sacc formation. The initial rate studies were meant to do two things, define the first and second order rate constants and determine whether Lys adds last. The pH dependence of V/K_{Lys} and the isotope effects on V and V/K_{Lys} were measured assuming V/K_{Lys} includes the microscopic rate constants for the catalytic pathway, i.e., can add as the last substrate. The kinetic mechanism is generally known and defining its details is not important to the studies of the mutant enzymes.

A pair-wise analysis of the kinetics of K99M was carried out with one reactant maintained at saturation and the initial rate measured as a function of one of the remaining substrates at different fixed levels of the third. The Lys/NADH pair exhibited parallel lines with competitive substrate inhibition at high concentrations of Lys, Figure 2A. The patterns obtained varying Lys at different fixed concentrations of α -Kg, intersected to the left of the ordinate, Figure 2B, consistent with the sequential mechanism proposed for the WT enzyme. The double reciprocal plot obtained varying α -Kg at different fixed levels of NADH with Lys maintained at saturation exhibited a series of lines that intersect to the left of the ordinate, Figure 2C. Overall, data are consistent with an ordered kinetic mechanism with NADH binding first, followed by α -Kg and Lys; substrate inhibition by Lys is consistent with an E-NAD-Lys complex as found for the WT enzyme [6]. The proposed kinetic mechanism is identical to the kinetic mechanism proposed for the WT and C205S enzymes at high pH [6, 11]. The increased preference for binding α -Kg before Lys likely derives from the very poor affinity for Lys ($K_m = K_d$, see 4.1 below). Although $K_{\alpha-Kg}$ also increases by almost 90-fold, it still has greater affinity than Lys.

The initial velocity pattern obtained for D319A, varying NADH and α -Kg at a fixed saturating concentration of Lys, gave parallel lines at low concentrations of α -Kg, but exhibited competitive substrate inhibition vs. NADH at high concentrations; data suggest binding of α -Kg to E (data not shown). A structural analog of α -Kg, OG, was uncompetitive against NADH. The intercept inhibition constant (K_{ii}) for OG was 4.0 ± 0.5 mM. On the basis of the limited data obtained, data for D319A are consistent with the mechanism of WT and C205S at neutral pH, i.e., binding of NADH to E, followed by random addition of Lys and α -Kg.

Kinetic parameters obtained for both mutant enzymes are summarized in Table 2. For the K99M mutant enzyme, $V_2/K_{Lys}E_t$ decreased about 175-fold, while $V/K_{\alpha-Kg}$ and V/K_{NADH} decreased at least 125- and 34-fold, respectively. The substrate inhibition constant (K_{iB}) for Lys for K99M was 1140 ± 160 mM. For the D319A mutant enzyme, V/K_{NADH} decreased 72-fold.

3.3 pH studies

The pH dependence of kinetic parameters was determined for D319A in the direction of Sacc formation at 25°C. Results are shown in Figures 3 (A–C). V_2/E_t was qualitatively and quantitatively similar to that of C205S [11]. It decreases from a pH independent value of $(132 \pm 8) s^{-1}$ to a lower pH independent value of $(12.6 \pm 0.1) s^{-1}$. The pK_a of the group indicated in the D319 V_2/E_t profile is about 6.8.

$V_2/K_{NADH}E_t$ for D319A decreases at low and high pH with limiting slopes of +1 and -1, giving pK_a values of 5.9 ± 0.1 and 9.1 ± 0.1 . The pH independent value of $V_2/K_{NADH}E_t$ was $(9.0 \pm 0.7) \times 10^5 M^{-1} s^{-1}$. The $V_2/K_{Lys}E_t$ for D319A also decreased at low and high pH giving pK_a values of 6.3 ± 0.2 and 8.5 ± 0.2 , perturbed by a little more than one pH unit compared to those observed for C205S [11]. The pH independent value of $V_2/K_{Lys}E_t$ is $(3.2 \pm 0.6) \times 10^4 M^{-1} s^{-1}$. The pH dependence of the kinetic parameters for K99M could not be measured due to very high K_m values above or below pH 7.0.

3.4 Kinetic isotope effects

Primary deuterium kinetic isotope effects were measured by direct comparison of initial rates as a function of Lys concentrations at pH 7.3 for K99M and at pH 9 for D319A. Both mutant proteins exhibited finite effects.

Solvent kinetic deuterium isotope effects were measured by direct comparison of the initial rates as a function of Lys concentration in H_2O and D_2O in the pH(D) independent range of the V and V/K pH-rate profiles. Solvent isotope effects for both mutant enzymes were measured at pH 7.0.

Multiple isotope effects were measured in H_2O and D_2O using NADD as the dinucleotide substrate in order to examine whether the substrate and solvent isotope effects reflect the same or different steps. The isotope effect obtained for K99M is on V/K_{Lys} only, since it was not possible to maintain saturating concentrations of NADH(D). Data are summarized in Table 3.

4 Discussion

4.1 K99M mutant enzyme

The mutation of K99 to methionine has eliminated a positive charge from the active site and resulted in the loss of a side chain that was capable of participating as a hydrogen bond donor. In the semi-empirical model, Figure 1, this residue interacts via hydrogen bonding

with Sacc and other neighboring residues. The values for $V_2/K_{\text{Lys}}E_t$ and $V_2/K_{\alpha\text{-Kg}}E_t$ are decreased by about two orders of magnitude, while $V_2/K_{\text{NADH}}E_t$ is decreased by an order of magnitude. However, V/E_t is not significantly affected. Thus, once all reactants are bound, the catalytic pathway does not appear to be much affected by the mutation. The proposed kinetic mechanism for the K99M mutant enzyme requires NADH bound prior to $\alpha\text{-Kg}$ or Lys, and in this case $V_2/K_{\text{NADH}}E_t$ is equal to k_I , the on-rate constant for binding NADH. An effect on the on-rate constant for NADH upon mutating K99 to M indicates a change in the active site of free enzyme compared to C205S, even though K99 does not directly interact with the dinucleotide substrate. Likewise, changes in the values of $V_2/K_{\text{Lys}}E_t$ and $V_2/K_{\alpha\text{-Kg}}E_t$, are consistent with increases in the K_d values for Lys from E-NADH- $\alpha\text{-Kg}$ -Lys and $\alpha\text{-Kg}$ from E-NADH- $\alpha\text{-Kg}$. According to the model presented in Figure 1, K99 directly interacts with the α -carboxylate of Lys, and the side chain of E122, so the decrease in the affinity of Lys is understandable. However, no such direct interaction with NADH or $\alpha\text{-Kg}$ is indicated, and there must be some other reason for the decreased affinity of these two reactants. Elimination of a lysine changes the balance of charge in the site, with more negatively charged than positively charged side chains. This may result in initial binding of the nicotinamide ring in a position different than would be expected in C205S, and once NADH is bound, weaker binding of $\alpha\text{-Kg}$ because of the position of the nicotinamide ring and the increase in negative charge in the site. Linked to this is generating the proper catalytic conformation, with the loss of K99 making it more difficult to generate the correct conformation at each step.

The isotope effects on V and V/K_{Lys} are identical for the C205S mutant enzyme [11], indicating K_{Lys} is equal to the K_d for Lys from the E-NADH- $\alpha\text{-Kg}$ -Lys quaternary complex [19]. Equality of isotope effects on V and V/K has been observed for almost all of the mutant enzymes prepared to date [13, unpublished data]. In addition, isotope effects are pH independent over the pH range 5–8 [10]. Taken together, data suggest the catalytic pathway that includes oxidative deamination limits the overall rate of the reaction for WT and the C205S mutant enzymes [10]. An increase in $D(V/K_{\text{Lys}})$ and decreases in V and V/K_{Lys} are observed, consistent with rate limitation by the catalytic pathway. As a result, K_{Lys} will be equal to K_d . The free energy of binding can thus be calculated for both enzymes ($\Delta G^{\circ} = -RT \ln K_{\text{eq}} = -RT \ln(1/K_d)$, where $1/K_d$ is the association constant, K_s) and these data corroborate conclusions made above using the second order rate constants. The K_s values estimated from the data in Table 2, for C205S and the K99M mutant enzymes, are $1.1 \times 10^3 \text{ M}^{-1}$ and 10.1 M^{-1} , respectively, giving values of -4.16 and -1.37 kcal/mol , respectively. The contribution of K99 to binding Lys, $\Delta \Delta G^{\circ}$, is thus 2.79 kcal/mol , more than half of the total binding energy for Lys. Similar calculations can be carried out for $\alpha\text{-Kg}$, assuming K_m values are equal to K_d . This is a reasonable assumption given the near equality in DV and $D(V/K_{\alpha\text{-Kg}})$ for WT and C205S [6, 11], and the 100-fold decrease in $V/K_{\alpha\text{-Kg}}$ for K99M compared to C205S. Calculated $1/K_d$ values for $\alpha\text{-Kg}$ from the E-NADH- $\alpha\text{-Kg}$ ternary complex are 9.1×10^3 and 114 M^{-1} , respectively, for C205S and C205S/K99M, giving ΔG° values of -5.4 and -2.8 kcal/mol , respectively, and a $\Delta \Delta G^{\circ}$ of 2.6 kcal/mol . The kinetic mechanism of SDH is ordered with NADH binding to free enzyme [6], and K_{NADH} is equal to the K_d for the E-NADH complex, which is equal to K_{NADH} . Values for C205S and K99M are 5×10^4 and $3 \times 10^3 \text{ M}^{-1}$, respectively, giving ΔG° values of -6.42 and -4.75 kcal/mol , respectively, and a $\Delta \Delta G^{\circ}$ of 1.67 kcal/mol . As suggested above, the change in free energy of binding almost certainly reflects binding to give the correct conformation.

For C205S, the primary deuterium isotope effect reports on the rate limitation of the hydride transfer step, while the solvent kinetic deuterium isotope effect reports on the rate limitation of the hydride transfer and imine formation steps, Scheme 2 [10]. For the wild type enzyme, the small primary deuterium isotope effects suggested hydride transfer contributes only slightly to rate limitation, while the substantial solvent deuterium effect, which decreases

upon deuteration of the substrate, suggests the solvent effect reflects the imine formation step. Repeating the primary deuterium isotope effect in D₂O further suggested that the hydride transfer step also exhibited a solvent deuterium effect [10].

Data for K99M provide information on the contribution of K99 to catalysis. The primary deuterium effect for K99M increases substantially to 2.2 compared to 1.3 for C205S, while the solvent deuterium effect decreases to 1.6 compared to 2.6 for C205S. The 175-fold decrease in V/K_{Lys} with less than a 2-fold change in V suggests that if substrate is sticky for C205S, it is no longer for K99M, and this may be the reason for the change in the isotope effects, i. e., a decrease in the value of c_f . However, if this were the case, both isotope effects, primary deuterium and solvent deuterium, would be expected to increase. Changes in the isotope effects in opposite directions as observed suggests a change in the partitioning of the imine formed from the condensation of Lys and α -Kg. Consider mechanism 8 for SDH under conditions of saturating concentrations of NADH and α -Kg. In mechanism 8, k_5 and k_6 represent the rate constants for addition to and release of lysine from the E-NADH- α -Kg-Lys complex, k_7 and k_8 represent the rate constants for imine formation, k_9 and k_{10} represent the rate constants for hydride transfer, and k_{11} and k_{13} represent the rate constants for release of Sacc and NAD, respectively. The E-NADH-imine complex, once formed can partition toward Sacc via the hydride transfer step, or toward the E-NADH- α -Kg-Lys complex via the hydrolysis of the imine. Equations for the primary deuterium and solvent deuterium kinetic isotope effects are given in equations 9 and 10.

(8)

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$$D \left(\frac{V}{K_{Lys}} \right) = \frac{Dk_9 + \left(\frac{k_9}{k_8} \right) \left(1 + \frac{k_7}{k_6} \right) + D K_{eq} \left(\frac{k_{10}}{k_{11}} \right)}{1 + \left(\frac{k_9}{k_8} \right) \left(1 + \frac{k_7}{k_6} \right) + \left(\frac{k_{10}}{k_{11}} \right)} \quad (9)$$

$$^{D2O} \left(\frac{V}{K_{Lys}} \right) = \frac{^{D2O}k_7 + \left(\frac{k_7}{k_6} \right) + ^{D2O}K_{eq} \left(\frac{k_8}{k_9} \right) \left(1 + \frac{k_{10}}{k_{11}} \right)}{1 + \left(\frac{k_7}{k_6} \right) + \left(\frac{k_8}{k_9} \right) \left(1 + \frac{k_{10}}{k_{11}} \right)} \quad (10)$$

There is evidence that Lys is slightly sticky in the E-NADH- α -Kg-Lys complex, so k_7/k_6 , the external forward commitment, c_{fex} , is finite and cannot be ignored. The ratio k_9/k_8 reflects partitioning of the E-NADH-imine complex. Since the imine is formed prior to hydride transfer, partitioning of the E-NADH-imine complex occurs prior to reduction and is included in c_f , the internal c_{fin} . If the imine partitions more toward hydride transfer, Scheme 2, the k_9/k_8 ratio will be high and a small deuterium isotope effect will be observed as found for C205S [11], while if the opposite is true, the k_9/k_8 ratio will be low and the isotope effect will be higher as found for K99M. In the case of the solvent deuterium kinetic isotope effect, the imine is formed in the isotope sensitive step and partitioning of the E-NADH-imine complex occurs after formation of the imine, and is included in the c_r term. If the imine partitions more toward hydride transfer, the k_8/k_9 ratio will be low and a large solvent deuterium isotope effect will be observed as found for C205S [11], while if the opposite is true, the k_8/k_9 ratio will be high and the isotope effect will be lower as found for K99M. The multiple isotope effect, $^{D2O}(V/K_{Lys})_D$, shows no or very little change compared to the solvent isotope effect, $^{D2O}(V/K_{Lys})$. Thus, as suggested for the wild type and C205S

enzymes [10, 11], the multiple isotope effect reflects isotope effects on imine formation and on the hydride transfer step, accounting for the increase in the value of 1.6 measured for $^{D_2O}(V/K_{Lys})$ to 2.0.

The change in the partition ratio of the E-NADH-imine complex, which favors hydride transfer for C205S to one that favors imine hydrolysis for K99M, likely reflects a change in the bound conformation of the imine. Lysine 99 interacts directly with the α -carboxylate of the substrate Lys, Figure 1, and elimination of K99, replacing it with the hydrophobic methionine, will almost certainly result in a reorientation of bound substrate. The decrease in the rate of hydride transfer relative to imine hydrolysis could result in a lengthening of the reaction coordinate for hydride transfer, and/or a nonlinear reaction coordinate for the hydride transfer step.

4.2 D319A mutant enzyme

Changing D319 to A results in less negative charge in the active site, and an increase in the net positive charge. Although slight, 3- to 6-fold decreases were observed for V , V/K_{Lys} , and $V/K_{\alpha-Kg}$, the only significant change observed was about a 100-fold decrease in the second order rate constant $V/K_{NADH}E_t$, suggesting the residue is involved in binding NADH in the active site. This result is not surprising. In Figure 1 the carboxamide side chain of the nicotinamide ring is shown near the carboxylate of D319, and elimination of the D319 side chain might be expected to decrease the affinity for the dinucleotide substrate. The free energy of binding of NADH for C205S and D319A can be estimated from the respective values of K_{NADH} , as above for K99M (assuming $K_{NADH} = K_{iNADH}$), giving $\Delta G^{0'}$ values of -6.83 and -4.97 kcal/mol. The calculated $\Delta\Delta G^{0'}$ value is 1.86 kcal/mol, about the same as the contribution of K99 to NADH binding.

Isotope effects measured for D319A were smaller than those measured for C205S. The primary deuterium effect on the hydride transfer step is lower as is the solvent deuterium effect, suggesting that either Lys has become stickier or some other step that is insensitive to isotopic substitution has become slower. The K_m for Lys for D319A is about the same as that obtained for C205S, Table 2, and the K_m is equal to K_d [11]. In addition, $^DV = ^D(V/K_{Lys})$, suggesting it is not Lys stickiness that has changed, but a non-isotope dependent step has become slower. The slow step is likely a conformational change elicited upon binding of NADH. This suggestion is quite reasonable. There is a requirement for binding of the dinucleotide prior to Lys and α -Kg to set up the site for optimum binding of the two reactants, and the binding of NADH is different in the D319A mutant enzyme. Data further suggest that the interaction between the carboxamide side chain of the nicotinamide ring and D319 is important in eliciting this conformational change. The slight increase in the solvent deuterium effects with deuterated substrate, $^{D_2O}(V/K_{Lys})_D$, $^{D_2O}V_D$, compared to that with unlabeled substrate, $^{D_2O}(V/K_{Lys})$, ^{D_2O}V , likely reflects the isotope effect on the hydride transfer step.

The pH dependence of kinetic parameters for D319A is very similar to that of C205S [11]. As discussed in Results, the V/E_t pH-rate profile is qualitatively and quantitatively identical to that of C205S, with a partial change observed as the pH is increased, proposed to reflect a pH-dependent isomerization of free enzyme [11]. We will concentrate on the differences observed as a result of mutating D319. Differences are observed in the V/K_{NADH} and V/K_{Lys} profiles. The $V_2/K_{Lys}E_t$ for D319A decrease at low and high pH and is qualitatively identical to that of C205S [11]. However, pK_a values of about 6.3 and 8.5 are obtained for D319A compared to values of 7 and 8 for C205S. The 0.4–0.7 pH unit shift in the pK_a values to lower and higher pH, likely results from the change in charge in the active site as a result of elimination of the negative charge associated with the D319 side chain. The most dramatic change in the pH-rate profiles is observed for $V/K_{NADH}E_t$. The pH-rate profile exhibits a

decrease at high pH, giving a pK_a of 9.1, identical to the pH dependence observed for C205S [11]. The group with a pK_a of 9.1 was proposed to reflect a lysine or arginine required protonated for optimum binding of NADH [10]. However, $V/K_{NADH}E_t$ also decreases at low pH giving a pK_a of about 5.9 and this group is not observed with C205S. The kinetic mechanism of SDH is ordered with NADH binding prior to Lys or α -Kg, so the pK_a of 5.9 must reflect a binding group for NADH in free enzyme. There is a possibility that the group is catalytic if the kinetic mechanism changed to allow NADH to bind as the last substrate, but the uncompetitive inhibition pattern obtained with OG, an analog of α -Kg, suggests the kinetic mechanism is still ordered with NADH binding to E. The pH dependence of $V/K_{NADH}E_t$ thus reflects groups important for binding NADH. The pH dependence of $V/K_{NADH}E_t$ thus reflects groups important for binding NADH. The effect of the group with a pK_a of 5.9 is unmasked in the D319A mutant enzyme, but its identity is at this point unknown.

4.3 Conclusions

Data obtained for both K99M and D319A support a role for each in substrate binding. Lysine 99 contributes 2.79 kcal/mol and 2.6 kcal/mol to Lys and α -Kg binding, and 1.85 kcal/mol to binding NADH, respectively. Aspartate 319 contributes 1.86 kcal/mol to NADH binding, a contribution identical to that of K99. On the basis of the semi-empirical model shown in Figure 1, it is easy to reconcile the contribution of K99 to Lys binding and D319 to NADH binding since there are close interactions with the reactants.

The role of K99 in binding α -Kg and NADH, however, suggests a more general role for K99 in determining the integrity of the active site in the Michaelis complex. In this regard, both residues do more than simply contribute to binding of reactants. Replacement of K99 with M also results in a change in the partition ratio of the E-NADH-imine complex. In C205S, the complex favors E-NAD-Sacc formation, while in the K99M mutant enzyme the E-NADH- α -Kg-Lys complex is favored. This change in partition ratio is consistent with a change in the conformation of Lys and α -Kg and/or a change in orientation of the imine, once formed, with respect to the nicotinamide ring of NADH. The change in partition ratio is also consistent with the proposed role of K99 in determining the integrity of the active site in the Michaelis complex. Also consistent with this suggestion is the fact that K99 is also within hydrogen bond distance to E122, which is within hydrogen bond distance to the α -amine of Lys. Loss of the K99-E122 interaction will certainly have an effect on the conformation of bound reactants. As pointed out above, D319 also aids in giving the correct conformation of the active site upon binding of NADH. This is important for favorable binding of Lys and α -Kg.

Highlights

Mutating K99 to M decreases affinity for lysine, α -ketoglutarate, and NADH

Partitioning of the imine intermediate favors imine hydrolysis in the K99M mutant

Mutating D319 to A greatly weakens NADH binding

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Figure 1.

Semi-empirical model of the E-NAD-Sacc complex of SDH from *Saccharomyces cerevisiae*. The figure is obtained from a superposition of the structures of SDH with sulfate (2QRJ), oxalylglycine (2QRL), and AMP (2QRK) bound [9]. The NMN moiety of NAD and the Lys moiety of Sacc are built-in by hand. The nicotinamide ring of NAD is shown near D319. Lys99 donates hydrogen bonds to the α -carboxylate of the Lysine moiety of Sacc, and E122. The figure was constructed using PyMol [18].

Figure 2.

Initial Velocity Patterns for the K99M Mutant Enzyme. (A). Double reciprocal plot of initial rate as a function of the concentration of NADH as shown, at different fixed levels of lysine: 10 mM ($\blacklozenge$); 13.3 mM ($\blacktriangle$); 19.8 mM ($\bullet$); 39 mM ($\blacklozenge$) and 1.2 M ($\blacksquare$). The concentration of α -Kg was fixed at 100 mM ($> 10K_{\alpha\text{-Kg}}$). Data exhibit competitive substrate inhibition by lysine. The points are experimental, while the lines are based on a fit to eq 3. (B). Double reciprocal plot of initial rate as a function of the concentration of Lys, shown at different fixed levels of α -Kg, 2.5 mM ($\blacklozenge$); 3.7 mM ($\blacktriangle$); 7.14 mM ($\bullet$) and 100 mM ($\blacksquare$). The concentration of NADH was fixed at 1.5 mM ($\sim 7.5K_{\text{NADH}}$). The points are experimental, while the lines are based on a fit to eq. 1. (C). Double reciprocal plot of initial rate as a function of the concentration of α -Kg, shown at different fixed levels of NADH, 0.008 mM ($\blacklozenge$); 0.01 mM ($\blacksquare$); 0.024 mM ($\blacktriangle$) and 1.2 mM ($\bullet$). The concentration of Lys was fixed at 1.2 M ($12K_{\text{Lys}}$). The points are experimental, while the lines are based on a fit to eq. 1.

Figure 3. pH Dependence of Kinetic Parameters for the D319A Mutant Enzyme in the Direction of Sacc Formation. Data were obtained at 25°C for V_2/E_t (A), $V_2/K_{Lys}E_t$ (B) and $V_2/K_{\alpha-Kg}E_t$ (C). The points are the experimentally determined values, while the curves are theoretical based on fits of the data using eq. 5 for B, C and eq. 7 for A.

Scheme 1.

Chemical Mechanism Proposed for Saccharopine Dehydrogenase. The reaction begins with the E-NAD-Sacc complex (I), and proceeds via the following intermediates. Imine intermediate (II), neutral carbinolamine intermediate (III), protonated carbinolamine intermediate (IV), product E-NADH-α-Kg-Lys complex with a neutral lysine ε-amine (V), and the product E-NADH-α-Kg-Lys complex with a protonated lysine ε-amine (VI). Stereochemistry of intermediates III and IV has not been established experimentally.

Table 1

DNA sequence for forward and reverse PCR primers.

Primers ^a	DNA sequence from 5' to 3'
K99M-F	5'-TTTGCTCACTGCTACAT TGG ACCAAGCTGGGTGGC-3'
K99M-R	5'-TGCCACCCAGCTTGGTCCATGTAGCAGTGAGCAAAC-3'
D319A-F	5'-ATTATCTGTCATCTCTAT TTG CTCACTTGCCTTCTTTGCTGC-3'
D319A-R	5'-GCAGCAAAGAAGGCAAGTGAGCA AT AGAGATGACAGATAAT-3'

Names of the primers used to mutate the *LYSI* cDNA, are in the left column and “F” and “R” stand for forward and reverse direction, respectively. The mutated codon is indicated in bold letters.

Table 2

Kinetic parameters for C205S, K99M and D319A mutant enzymes.

Kinetic parameters	C205S ^a	K99M	D319A
V_2/E_t (s ⁻¹) ^b	106	68 ± 4	35 ± 5
$V_2/K_{\text{NADH}}E_t$ (M ⁻¹ s ⁻¹)	1.1×10 ⁷	(3.5 ± 0.5) × 10 ⁵	(1.5 ± 0.5) × 10 ⁵
$V_2/K_{\alpha\text{-Kg}}E_t$ (M ⁻¹ s ⁻¹)	9.7 × 10 ⁵	(7.8 ± 0.1) × 10 ³	(1.7 ± 0.2) × 10 ⁵
$V_2/K_{\text{Lys}}E_t$ (M ⁻¹ s ⁻¹)	1.2 × 10 ⁵	(6.8 ± 0.3) × 10 ²	(2.9 ± 0.4) × 10 ⁴
K_{NADH} (mM)	0.01	0.20 ± 0.01	0.23 ± 0.01
K_{Lys} (mM)	0.89	99 ± 4	1.2 ± 0.2
$K_{\alpha\text{-Kg}}$ (mM)	0.11	8.8 ± 0.4	0.21 ± 0.02
K_{INADH} (mM)	0.02	0.31 ± 0.06	ND

^aThe C205S mutant enzyme is the reference for all mutant studies. Data were obtained in the direction of Sacc formation at 25°C and pH 7.2 [11]. ND is not determined.

^b V_2/E_t values obtained from different pairwise analyses were, within error, equal.

Table 3

Summary of the isotope effects for K99M and D319A.

Parameter	C205S ^a	K99M	D319A
^D (V)	(pH 9) 1.3 ± 0.2	(pH 7) ND ^b	(pH 9) 1.13 ± 0.01
^D (V/K _{Lys})	1.3 ± 0.2	2.22 ± 0.03	1.13 ± 0.01
^{D2O} (V)	2.6 ± 0.4	ND ^b	1.24 ± 0.03
^{D2O} (V/K _{Lys})	2.6 ± 0.4	1.6 ± 0.1	1.24 ± 0.03
^{D2O} (V) _D	1.8 ± 0.1	ND ^b	1.48 ± 0.10
^{D2O} (V/K _{Lys}) _D	1.8 ± 0.1	2.0 ± 0.6	1.48 ± 0.10

^aThe C205S mutant enzyme is the reference for all mutant studies. Data were obtained in the direction of Sacc formation at 25°C [11]

^bThe isotope effect on V was not determined because of the high values of K_{NADH} and $K_{\alpha-Kg}$.