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Probing the Chemical Mechanism of Saccharopine Reductase from *Saccharomyces cerevisiae* Using Site-Directed Mutagenesis

Ashwani K. Vashishtha^b, Ann H. West^{a,*}, and Paul F. Cook^{a,*}

^aDepartment of Chemistry and Biochemistry, University of Oklahoma, 101 Stephenson Blvd., Norman, Oklahoma 73019

^bDepartment of Chemistry and Biochemistry, University of Colorado, Boulder, 3415 Colorado Ave, JCS Biotech Bldg. 530, Boulder, Colorado 80309

Abstract

Saccharopine reductase catalyzes the reductive amination of L- α -amino adipate- δ -semialdehyde with L-glutamate to give saccharopine. Two mechanisms have been proposed for the reductase, one that makes use of enzyme side chains as acid-base catalytic groups, and a second, in which the reaction is catalyzed by enzyme-bound reactants. Site-directed mutagenesis was used to change acid-base candidates in the active site of the reductase to eliminate their ionizable side chain. Thus, the D126A, C154S and Y99F and several double mutant enzymes were prepared. Kinetic parameters in the direction of glutamate formation exhibited modest decreases, inconsistent with the loss of an acid-base catalyst. The pH-rate profiles obtained with all mutant enzymes decrease at low and high pH, suggesting acid and base catalytic groups are still present in all enzymes. Solvent kinetic deuterium isotope effects are all larger than those observed for wild type enzyme, and approximately equal to one another, suggesting the slow step is the same as that of wild type enzyme, a conformational change to open the site and release products (in the direction of saccharopine formation). Overall, the acid-base chemistry is likely catalyzed by bound reactants, with the exception of deprotonation of the α -amine of glutamate, which likely requires an enzyme residue.

Keywords

Saccharopine Reductase; site-directed mutagenesis; initial rate studies; pH-rate profiles; isotope effects; viscosity

Saccharopine reductase (SR¹) [saccharopine dehydrogenase (L-glutamate forming), EC 1.5.1.10] catalyzes the penultimate step in the α -amino adipate pathway for the *de novo*

*Corresponding authors: pcook@ou.edu, Tel: 405-623-2970, awest@ou.edu, 405-325-1529.

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synthesis of L-lysine in fungi (1-3), the reductive amination of L- α -aminoadipate- δ -semialdehyde with L-glutamate to give saccharopine, eq. 1 (1).

(1)

An ordered kinetic mechanism has been proposed for SR on the basis of initial velocity studies in the absence and presence of product and dead-end inhibitors (4). The reduced dinucleotide substrate binds to enzyme first followed by L- α -aminoadipate- δ -semialdehyde (AASA), which adds in rapid equilibrium prior to L-glutamate; saccharopine is released prior to NADP.

On the basis of the pH dependence of the kinetic parameters, primary deuterium kinetic isotope effects and solvent deuterium kinetic isotope effects, a chemical mechanism was proposed for SR from *Saccharomyces cerevisiae* (Scheme 1) (5). The proposed mechanism suggested two groups are involved in the acid-base chemistry of the reaction. An enzyme group with a pK_a of 5.6 accepts a proton from the α -amine of glutamate to generate the neutral amine that can act as a nucleophile (II in Scheme 1). The α -amine of glutamate attacks the carbonyl of the semialdehyde to generate the carbinolamine, which is protonated by a second enzyme group with a pK_a of about 7.8-8.

Crystal structures of the apoenzyme of SR from *Saccharomyces cerevisiae* was solved to 1.7 Å resolution (7), and that from *Magnaporthe grisea* was solved to 2.0 Å resolution (6). The ternary, E-NADPH-saccharopine, complex of SR from *M. grisea* has also been solved to 2.1 Å, respectively (6). The SR from *M. grisea* shows an overall sequence identity and similarity of 63% and 78%, respectively, to the enzyme from *S. cerevisiae*. Comparison of the active site superimposed structure of SR from *M. grisea* and *S. cerevisiae* shows that positions of all residues in the active site are completely conserved. Ionizable residues that could play a role in the acid-base mechanism of SR are D126, C154 and/or Y99. A multiple sequence alignment of SR from *S. cerevisiae*, *M. grisea*, *Schizosaccharomyces pombe*, *Aspergillus fumigatus*, *Candida albicans*, and *Cryptococcus neoformans* indicate indicated all three residues are highly conserved (data not shown). At least two residues, D126 and Y99, may be in position to act as a base, accepting a proton from the α -amine of glutamate (II in Scheme 1), and an acid, protonating the leaving hydroxyl as the carbinolamine is converted to the imine (IV to V in Scheme 1). The distance between the closest oxygen of

¹Abbreviations: AAA, α -aminoadipate; SR, saccharopine reductase (saccharopine dehydrogenase: L-glutamate forming); NADP, nicotinamide adenine dinucleotide phosphate (the + charge is omitted for convenience); NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); Sacc, L-saccharopine; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Mops, 3-(*N*-Morpholino)-propanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; DCl, deuterium chloride; NaOD, sodium deuterioxide; D₂O, deuterium oxide; L- α -aminoadipate- δ -semialdehyde, AASA

D126 and the secondary amine nitrogen of saccharopine is long enough (5.57 Å) to accommodate a water molecule. Cysteine 154, although conserved, points away from the active site into the protein structure.

Authors of the *M. grisea* structure paper suggested the reaction proceeded once reactants were bound to the active site, catalyzed by reactant functional groups (6). The proposed mechanism is shown in Scheme 2. A recent computational study (8) making use of QM/MM-based computational studies considered each of the two proposed mechanisms, but their results were consistent with the mechanism shown in Scheme 2, which will be considered below in **DISCUSSION**.

In this paper, site-directed mutagenesis was used to change D126, C154 or Y99 to A, S, and F, respectively. Mutant enzymes were characterized using initial velocity studies, the pH dependence of the kinetic parameters, and isotope effects. Data are generally consistent with the mechanism proposed in Scheme 2. A re-investigation of the data used to propose the mechanism in Scheme 1 is undertaken in light of these conclusions.

MATERIALS AND METHODS

Chemicals and Enzymes

L-Saccharopine, L-glutamate, *Thermoanaerobacter brockii* alcohol dehydrogenase, and yeast aldehyde dehydrogenase were from Sigma. β -NADPH and β -NADP were purchased from USB. Mes, Mops, Ches and Hepes were from Research Organics, and D₂O (99 atom% D) was from Cambridge Isotope Laboratories, Inc. The α -amino adipate- δ -semialdehyde was synthesized as described previously (6). The 4R-NADPD was prepared according to Viola et al. (9). All other chemicals were of the highest grade available and were used as purchased.

Cell growth, expression, and purification of the WT and mutant enzymes were carried out as reported previously (6, 7).

Site Directed Mutagenesis

The D126A, C154S, Y99F, D126A/C154S, D126A/Y99F and C154S/Y99F mutant enzymes were prepared, using the Quick Change site-directed mutagenesis kit (Stratagene), in accordance with the recommendations of the manufacturer. Mutant genes were prepared using the pET-16b plasmid, which houses the *S. cerevisiae* *LYS 9* gene as a template. Forward and reverse primers used for preparation of the mutated genes are given in Table 1. Double mutant genes, D126A/C154S, D126A/Y99F and C154S/Y99F, were prepared using the single mutant genes for D126A, and C154S as a template and using forward and reverse primers for C154S and Y99F.

All mutant genes were sequenced at the Oklahoma University Health Science Center Gene Sequencing Facility and the nucleotide sequences of the mutant enzymes were confirmed. Resulting plasmids were used to transform BL21 DE3-RIL competent cells and all mutant proteins were expressed as previously reported (4, 5).

Initial Velocity Studies

The initial rate was measured as the change in absorbance at 340 nm with time, reflecting the change in NADPH concentration (ϵ_{340} , 6,220 M⁻¹cm⁻¹), using a Beckman DU-640 spectrophotometer. Reactions were carried out in quartz cuvettes with a path length of 1 cm in a final volume of 0.5 mL containing 200 mM buffer, and variable concentrations of substrates. Assays were carried out at 25 °C.

In order to determine whether the kinetic parameters of a given mutant enzyme changed compared to WT, initial rate studies were carried out in the direction of formation of L-saccharopine at pH 7.0 and in the direction of formation of L-glutamate at pH 9.0. Initial rate data in the direction of saccharopine formation were measured by varying one substrate at fixed saturating concentrations of the other two substrates. In the reverse reaction direction, initial rate data were collected at pH 9.0 as a function of NADP at different fixed concentrations of saccharopine.

pH Studies

The pH dependence of V_2/E_t , and $V_2/K_{Sacc}E_t$ were obtained for the D126A, C154S, Y99F, D126A-C154S, and D126A-Y99F mutant enzymes by measuring the initial rate as a function of saccharopine concentration with NADP maintained at saturation ($10K_{NADP}$). The activity of Y99F-C154S mutant enzyme was very low and a pH-rate profile was not obtained. The pH was maintained using the following buffers for the pH ranges indicated at a final concentration of 200 mM: Mes, 6.0-6.5; Mops, 6.5-7.5; Hepes, 7.5-8.5; Ches, 8.0-10.5. The pH was measured before and after the reaction and no significant change in pH was detected. In order to ensure that no effects of buffer were observed, data were obtained at the same pH when buffers were changed; no effects were observed.

Solvent Deuterium Kinetic Isotope Effect

In the direction of glutamate formation, solvent deuterium kinetic isotope effect studies were carried out for the D126A, C154S, Y99F, D126A-Y99F and D126A-C154S mutant enzymes. V_2/K_{Sacc} was measured at pH(D) 9.0 in 100% H₂O, and 100% D₂O with saccharopine as the variable substrate at a fixed concentration of NADP ($10K_m$). The solvent deuterium kinetic isotope effects, $^{D_2O}V_2$ and $^{D_2O}(V_2/K_{Sacc})$, are the ratios of V_2 and V_2/K_{Sacc} in H₂O and D₂O. For rates measured in D₂O, substrates (NADP and Sacc) 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 (10). Reactions were initiated by adding a small amount of each of the mutant enzymes in H₂O. The final D₂O concentration in the reaction mixture was ~95%.

Effect of Solvent Viscosity

In order to determine whether the finite solvent deuterium isotope effects observed resulted from an effect of increased solvent viscosity, 9% glycerol (w/v) was used as a viscosogen, generating approximately the same relative viscosity as 100% D₂O at 25°C, $\eta_{rel} = 1.24$ (11).

The V_2/K_{Sacc} was measured in the absence and presence of 9% glycerol, and the ratio of the two gives the effect of viscosity.

Data Analysis

Initial velocity data were first analyzed graphically as double reciprocal plots of initial rates vs. substrate concentration to determine data quality and the proper rate equation for data fitting. Data were then fitted to the appropriate rate equation using the Marquardt-Levenberg algorithm supplied with the EnzFitter program by BIOSOFT, Cambridge, U. K. and programs developed by Cleland (12). Kinetic parameters and their corresponding standard errors were estimated using a simple weighting method. Saturation curves for Sacc were fitted using eq. 2. Initial velocity data at pH 9.0 for the D126A mutant enzyme in the direction of formation of glutamate were fitted using eq. 3.

$$v = \frac{V A}{K_a + A} \quad (2)$$

$$v = \frac{V A B}{K_{ia} K_b + K_b A + K_a B + A B} \quad (3)$$

In eqs 2 and 3, v and V are the observed initial and maximum rates, respectively, A and B are substrate concentrations, K_a and K_b are Michaelis constants for substrates A and B, respectively, and K_{ia} is the dissociation constant for the EA complex.

pH-rate profiles were generated by plotting $\log V/K_E$ values as a function of pH to determine the appropriate rate equation for data fitting. pH rate-profiles that exhibited a slope of 1 at low pH and -1 at high pH were fitted using eq. 4, while those that exhibited a slope of 1 at low pH were fitted using eq. 5 (12).

$$\log y = \log \left[C / \left(1 + \frac{H}{K_1} + \frac{K_2}{H} \right) \right] \quad (4)$$

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

In eqs 4 and 5, y is the observed value of V/K as a function of pH, C is the pH-independent value of y , H is the hydrogen ion concentration, K_1 and K_2 represent acid dissociation constants for functional groups on the reactant or enzyme required in a given protonation state for optimal binding and/or catalysis.

RESULTS

Initial Velocity Studies

Initial velocity patterns were obtained in both reaction directions as discussed in the Methods section. In the direction of glutamate formation, data were obtained at pH 7 for the D126A and D126A-Y99F mutant enzymes, and kinetic parameters are summarized in Table

2. Decreases of about 10- and 20-fold in V_1/E_t are observed for the two mutant enzymes, respectively, while $V_1/K_{\text{Glu}}E_t$ was unchanged for D126A, and a 10-fold change was observed for the double mutant enzyme. The largest change was in $V_1/K_{\text{NADPH}}E_t$, with 75- and 292-fold decreases observed for D126A and D126A-Y99F. Overall, data suggest the residues play a role in binding of NADPH, but are otherwise not essential to the overall reaction.

In the direction of Sacc formation, data were obtained at pH 9 for the D126A, Y99F, and D126A-Y99F mutant enzymes, respectively, and kinetic parameters are summarized in Table 3. Changes in V_2/E_t of about 10-, 45-, and 300-fold are observed for the three mutant enzymes, suggesting the two residues are somewhat important to the overall reaction. Much larger decreases are observed in the second order rate constants, $V_2/K_{\text{NADP}}E_t$ and $V_2/K_{\text{Sacc}}E_t$ with the largest observed for the Y99F and D126A/Y99F mutant enzymes.

In the direction of Sacc formation, the C154S mutant enzyme gave modest decreases in all of the kinetic parameters with 7- to 17-fold decreases observed in the first and second order rate constants. The C154S-Y99F double mutation gave larger changes, with the largest decrease for both mutant enzymes observed for $V_1/K_{\text{NADPH}}E_t$, likely reflecting an effect on the binding of NADPH. In the direction of glutamate formation, however, changes are more substantial. The turnover number for the C154S mutant enzyme and the $V_2/K_{\text{NADP}}E_t$ decreased by two orders of magnitude, while $V_2/K_{\text{Sacc}}E_t$ decreased by more than three orders of magnitude. The double mutations, D126A-C154S and Y199F-C154S, exhibit a 3-4 order of magnitude decrease in the turnover number, close to the product of the changes observed for the single mutant enzymes, i.e., the C154S, D126A, and Y99F mutant enzymes. Decreases in $V_2/K_{\text{Sacc}}E_t$ are very large and range between 4 and 5 orders of magnitude. The significant decreases suggest a role of all three residues in catalysis, binding, and/or the structure of the active site.

pH Dependence of Kinetic Parameters

The pH dependence of kinetic parameters provides information on groups required in a given protonation state for optimum binding of reactant and/or catalysis (12). The pH dependence of V/K_{Sacc} was determined for the D126A, C154S, Y99F, D126A-Y99F, and D126A-C154S mutants are shown in Figures 2 and 3.

All pH-rate profiles, with the possible exception of that of the D126A-C154S mutant enzyme decrease at low and high pH with slopes of +1 and -1, respectively. Table 4 summarizes pK values and pH independent values for $V/K_{\text{Sacc}}E_t$.

Kinetic Isotope Effects

Initial rates were measured at saturating NADP, varying saccharopine around the pH(D) independent values of V_2/K_{Sacc} . The solvent deuterium kinetic isotope effects for D126A, C154S, Y99F, D126A-C154S, and D126A-Y99F mutant enzymes are summarized in Table 5. In all cases, finite isotope effects were observed that were about equal to and somewhat larger than those of WT (5).

Glycerol was used as a viscosogen for the mutant enzymes. Ratios of values of V_2/K_{Sacc} determined in H_2O and at a relative viscosity of 1.24 are within error equal. Therefore, the values of the observed solvent isotope effects, $^{D_2\text{O}}(V_2/K_{\text{Sacc}})$ for the mutant enzymes result from a classical isotope effect and not from an effect of the increased viscosity of 100% D_2O ($\eta_{\text{rel}} = 1.24$).

DISCUSSION

Initial Velocity Studies

There are few ionizable residues in the active site of SR near the substrate-binding site. Two of these are arginine residues that donate a hydrogen bond to carboxylates of Sacc, Fig. 1. In the view of the active site reactants bound as shown in the abortive *M. grisea* E-NADPH-Sacc ternary complex (6), D126 and Y100 are about 6 Å away from the secondary nitrogen of Sacc and C154 is pointed away from the active site toward the interior of the protein (Fig. 1). In order to determine whether any of these residues were directly involved in catalysis, they were changed to alanine, phenylalanine and serine, respectively, in the *S. cerevisiae* enzyme and characterized kinetically.

The mutations made resulted in enzymes with active site residues that could not catalyze the reaction if the residues were involved in catalysis. Kinetic parameters in the direction of Sacc formation exhibit modest decreases compared to WT, suggesting these residues are likely not acid-base catalytic residues in the overall reaction, Table 2. More substantial decreases are observed in the direction of Glu formation, Table 3, with most of the decrease in V ; note the much smaller increases in K_m and K_d . Results obtained for the double mutant enzymes are roughly multiplicative of the results from the individual mutations. For example, the product of the decreases in V for the D126A and Y99F single mutations in Table 3 approximate the decrease in V for D126A/Y99F. Data thus suggest no direct interaction of the individual residues in the active site with one another during the course of the reaction, i.e., the residues, in whatever function they have in the overall reaction, for the most part act independently of one another. Thus, the step(s) that limit the reaction at saturating concentrations of reactant are effected by mutating D126, Y99, and C154. The role of the residues mutated will be further considered below.

Interpretation of Solvent Deuterium Kinetic Isotope Effects

Kinetic and solvent deuterium isotope effects measured with WT SR were interpreted in terms of a slow conformational change to open the site and release products in the direction of Sacc formation or close the site once reactants are bound in the direction of Glu formation (5). As shown in Table 5, the SKIE on V_2 and V_2/K_{Sacc} measured for the mutant enzymes are, within error, identical to one another, and slightly larger than the effects obtained with the WT enzyme. Data are consistent with the same slow conformational change proposed for the WT enzyme, limiting the rate of the mutant enzymes.

Interpretation of pH Dependence of Kinetic Parameters

Finally, all of the pH-rate profiles shown in Figures 2 and 3, exhibit a decrease in rate at low and high pH and give estimated $\text{p}K_a$ values that are similar to those of WT, Table 4. Data are

again consistent with a lack of catalytic function for D126, Y99, and C154. Rather, changes in these residues very likely effects a pre-catalytic conformational change in the direction of Glu formation. As stated in RESULTS above, a significant decrease in V_1/K_{NADPH} suggests an additional effect of mutations on the binding of NADPH. Since NADPH is the first reactant bound in the direction of Sacc formation, properly bound NADPH would be important for the subsequent conformational change that occurs once reactants are bound. Thus, the effect on binding of NADPH is likely linked to the decrease in the rate of the pre-catalytic conformational change. A similar effect is observed (to a somewhat lesser extent for D126A) on V_2/K_{NADP} in the direction of Glu formation, Table 3.

Re-interpretation of the pH-rate Profiles for WT Saccharopine Reductase (5)

The pH-rate profiles obtained for WT enzyme were interpreted in terms of enzyme residues acting as general acid-base catalysts. On the basis of data presented in this study and the results of the QM-MM studies published previously (8), the chemical mechanism is auto-catalyzed by the bound reactants and the pH-rate profiles reflect pK_a values of the reactants. Thus, pH-rate profiles obtained in (5) will be re-interpreted in terms of Scheme 2, which outlines the mechanism proposed by Almasi et al. (8). Given the proposed ordered kinetic mechanism in which Glu adds last in the direction of Sacc formation and Sacc adds last in the direction of Glu formation, the V_1 , V_2 , V_1/K_{Glu} and V_2/K_{Sacc} are the parameters of interest since all include the rate constants for the chemical steps. The V/K pH-rate profiles exhibit pK_a s of reactant functional groups prior to binding of the final reactant and of the other bound reactants, while V will exhibit pK_a s of bound reactant functional groups. Thus, V_1 will exhibit pK_a s of bound AASA and Glu, respectively, and V_1/K_{Glu} will exhibit pK_a s of bound AASA and free Glu, while V_2 will exhibit pK_a s of bound Sacc and V_2/K_{Sacc} will exhibit pK_a s of free Sacc.

As shown in Scheme 2, the α -amine of Glu must be unprotonated to begin the reaction via a nucleophilic attack by the α -amine on the aldehydic carbon of AASA in the direction of Sacc formation. Thus, one of two scenarios must exist; either the enzyme selectively binds Glu with a neutral α -amine, or an enzyme group must accept a proton from the α -amine to start the reaction. The V_1/K_{Glu} pH-rate profile exhibits a pK_a of 5.6 on the acid side and a pK_a s of 7.9 and 8.5 on the basic side. The predominant forms of enzyme and reactant under these conditions are the E-NADPH-AASA complex and free Glu in solution. The group with a pK_a of 5.7 likely reflects an enzyme group that accepts a proton from the α -amine of Glu as it binds to enzyme. This group is not D126, because the D126A mutant enzyme exhibits small changes in the rate and pH dependence of the reaction. The groups observed on the basic side of the profile are assigned to the α amines of Glu and bound AASA. Thus, prior to I in Scheme 2, the α -amine of Glu is likely deprotonated by an enzyme residue to give I.

In the direction of Sacc formation, V_1 decreases at low and high pH giving pK_a s of 5.7 and 7.9, and these must reflect the α -amines of the bound forms of Glu and AASA. The pK_a of 5.7 is assigned to the α -amine of Glu and that of 7.9 assigned to the α -amine of AASA bound to enzyme. The low value, 5.7, of the pK_a assigned to the α -amine of Glu likely reflects placement of the α -amine into a nonpolar environment, or its close proximity to

another positively charged group within the active site. There is precedence for both of the above possibilities giving a decrease in the pK_a value of primary amines. Acetoacetate decarboxylase exhibits a pK_a of 6 for Lys115 in the active site as a result of its placement in a nonpolar environment (14). In addition, Lys256 in aspartate aminotransferase also exhibits a pK_a of 6 as a result of its proximity to the amine of pyridoxamine 5'-phosphate (15). Of the two possibilities, the latter is most likely, given the proximity of the α -amine of Glu to the α -amine of AASA, Fig. 1.

In the direction of Glu formation, V_2 decreases at low pH giving a pK_a of 7.2, which reflects the α -amine of the AASA moiety of Sacc. The V_2/K_{Sacc} decreases at low and high pH, giving pK_a s of 7.6 and 9.9, which reflect α -amine and secondary amine of Sacc, respectively. If data were collected to low enough pH in the previous study (5), an additional pK_a of about 5.7 would be expected for D126, which must accept a proton from the secondary amine (either directly or via a water molecule bridge).

Once reactants are bound, the reaction would be expected to proceed via Schemes 2. The α -amine of Glu is likely deprotonated by some enzyme residue. Then, as shown in Scheme 2, a nucleophilic attack by the α -amine of Glu on the aldehyde carbonyl of AASA occurs as in I, with the carbonyl oxygen protonated by the α -amine of AASA, which remains hydrogen-bonded to the hydroxyl of the newly formed carbinolamine. The carbinolamine hydroxyl accepts a proton from the carbinolamine nitrogen assisted by the α -carboxylate of the Glu moiety to give the neutral carbinolamine, II in Scheme 2. Water is eliminated to generate the imine, III in Scheme 2, followed by reduction of the imine, IV in Scheme 2, to give Sacc, V in Scheme 2.

Conclusions

Data are consistent with the acid-base mechanism proposed by the QM/MM studies (8), i.e., direct catalysis by bound reactants as suggested below.

1. The decrease in V/E_t and $V/K_{Glu}E_t$ was 40-fold for all mutant enzymes, suggesting that none are catalytically essential.
2. The V_2/K_{Sacc} pH-rate profiles for all mutant enzymes decrease at low and high pH, suggesting that acid and base catalytic residues are still present.
3. Solvent kinetic deuterium isotope effects are equal for all mutant enzymes, and slightly larger than those for wild type enzyme. The slow step proposed for the wild type enzyme, a conformational change to open the site to release Sacc is the same, but slower in all mutant enzymes.

The only step that cannot be accounted for in the QM/MM mechanism is the deprotonation of Glu in the direction of Sacc formation. We propose this may be carried out by an enzyme residue or that only Glu with a neutral α -amine can bind to enzyme.

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- D126, C154, and Y99 were changed alone and in pairs to give mutant enzymes
- Small changes in rate constants suggest residues are not catalytically essential
- pH-rate profiles are qualitatively similar to those of wild type enzyme
- Data are consistent with the intramolecular mechanism proposed from QM/MM studies
- $D_2O V$ and $D_2O(V/K)$ are identical and larger than those measured for wild type enzyme
- All residues mutated likely facilitate a conformational change to close the site

Scheme 1.

Proposed Enzyme Acid-base Chemical Mechanism for Saccharopine Reductase. **I:** Reactants are bound with the α -amines of Glu and AASA protonated. Reactants are in proximity to a general base and a general acid. **II:** The α -amine of Glu is deprotonated by the general base, B_2 . Nucleophilic attack by the α -amine of Glu on the aldehyde carbonyl occurs to give a protonated carbinolamine. The carbonyl oxygen is protonated by the general acid, B_1H . **III:** The carbinolamine is deprotonated by general base B_1 . **IV:** Water is eliminated with the aid of a general acid, B_1H to give the imine (**V**). **VI:** The secondary amine N of Sacc is protonated by B_2H to give the final product Sacc (**VII**).

Scheme 2.

Proposed Chemical Mechanism for Saccharopine Reductase with Catalysis by Bound Reactants. As Glu binds, its α -amine may be deprotonated by D126. **I:** Reactants are bound with the α -amine of Glu unprotonated and the α -amine of AASA protonated. Nucleophilic attack by the α -amine of Glu on the aldehyde carbonyl occurs to give a protonated carbinolamine. The carbonyl oxygen is protonated by the α -amine of AASA (**II**). **II:** The α -carboxylate of the Glu moiety acts in concert with the α -amine of AASA to generate the neutral carbinolamine. **III:** Water is eliminated from the carbinolamine to generate the imine. **IV:** Hydride transfer from NADPH to the imine carbon gives Sacc (**V**).

Figure 1.

Close-up View of the Active Site of Saccharopine Reductase from *M. grisea* (pdb 1E5Q). Residues that interact with saccharopine and hydrogen-bonding distances to the secondary amine nitrogen of saccharopine are shown. The following color scheme is used: C, green, O, red, N, blue; and S, orange. The figure was generated using the PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC..

Figure 2. pH Dependence of $V_2/K_{Sacc}E_t$. The pH-rate profiles are shown for: A. D126A; B. C154S; and C. Y99F. All data were obtained at 25°C. Points are experimental, while curves are based on a fit of the data to eq. 4.

Figure 3. pH Dependence of $V_2/K_{Sacc}E_t$. The pH-rate profiles are shown for: A. D126A-C154S; and B. D126A-Y99F. All data were obtained at 25°C. Points are experimental, while curves are based on a fit of the data to eq. 5 (A) or eq. 4 (B).

Table 1

Forward and Reverse Primers Used for Site-directed Mutagenesis

D126A _f	5'CGA AAT TGG GTT GGC TCC AGG TAT CGA CC3'
D126A _r	5'GGT CGA TAC CTG GAG CCA ACC CAA TTT CG3'
C154S _f	5'CTT GTC ATA CTC TGG TGG TTT ACC3'
C154S _r	5'GGT AAA CCA CCA GAG TAT GAC AAG3'
Y99F _f	5'CGT CAC TTC CTC TTT CAT CTC ACC TGC3'
Y99F _r	5'GCA GGT GAG ATG AAA GAG GAA GTG ACG3'

^a Subscript r and f reflect forward and reverse primers; mutated codons are in bold.

Table 2

Kinetic Parameters in the Direction of Saccharopine Formation at pH 7.0.

Parameter ^a	WT	D126A	D126A-Y99F	C154S	Y99F-C154S
V_i/E_t (s ⁻¹)	1.9 ± 0.1	0.100 ± 0.004 (19) ^a	0.080 ± 0.003 (24)	0.27 ± 0.02 (7)	0.050 ± 0.004 (38)
$V_i/K_{\text{NADPH}} E_t$ (M ⁻¹ s ⁻¹)	(1.7 ± 0.4) × 10 ⁵	(2.3 ± 0.2) × 10 ³ (75)	583 ± 31 (292)	(1.7 ± 0.1) × 10 ⁴ (10)	264 ± 18 (644)
$V_i/K_{\text{Glu}} E_t$ (M ⁻¹ s ⁻¹)	68 ± 16	67 ± 2 (1)	6.25 ± 0.50 (11)	4.0 ± 0.6 (17)	1.5 ± 0.3 (45)
K_{NADPH} (μM)	11.4 ± 2.6	44 ± 5 (4)	146 ± 13 (13)	164 ± 25 (14)	209 ± 35 (18)
K_{Glu} (mM)	28 ± 6	1.5 ± 0.1 (-19)	2.64 ± 0.02 (-11)	67 ± 2 (2.4)	33 ± 9 (1.1)
K_{IAASA} (mM)	0.31 ± 0.07	1.26 ± 0.08 (4)	7.4 ± 0.9 (24)	2.46 ± 0.02 (8)	2.3 ± 0.3 (7.4)

^aValues in () below the value is fold change compared to WT.

Table 3

Kinetic Parameters in the Direction of Glutamate Formation at pH 9.0.

Parameter ^a	WT	D126A	Y99F	D126A-Y99F	C154S	D126A-C154S	Y99F-C154S
V_2/E_t (s ⁻¹)	13 ± 1	1.4 ± 0.1 (9) ^a	0.29 ± 0.01 (45)	0.043 ± 0.004 (302)	0.12 ± 0.03 (112)	0.0028 ± 0.0005 (4643)	0.0014 ± 0.0004 (9285)
$V_2/K_{\text{NADP}}E_t$ (M ¹ s ⁻¹)	(8.5 ± 1.2) × 10 ⁴	(2.0 ± 0.1) × 10 ⁴ (4.2)	391 ± 24 (217)	110 ± 18 (772)	428 ± 80 (200)	4.1 ± 0.1 (20,730)	ND ^a
$V_2/K_{\text{succ}}E_t$ (M ¹ s ⁻¹)	(1.7 ± 0.3) × 10 ⁴	(6.7 ± 0.2) × 10 ³ (2.5)	51 ± 2 (331)	20 ± 2 (850)	6.7 ± 0.5 (2540)	0.88 ± 0.15 (19,320)	0.044 ± 0.002 (390,000)
K_{NADP} (mM)	0.15 ± 0.01	0.071 ± 0.005 (-2)	0.74 ± 0.05 (5)	0.39 ± 0.06 (2.5)	0.28 ± 0.06 (0.5)	0.68 ± 0.05 (4.5)	ND
K_{succ} (mM)	0.77 ± 0.12	0.21 ± 0.04 (4)	5.6 ± 0.5 (7)	2.1 ± 0.4 (3)	18.3 ± 7.2 (24)	3 ± 1 (4)	32 ± 10 (42)

^a Values in () below the value is fold change compared to WT; ND is not determined.

Table 4

Summary of pK_a Values for $V_2/K_{Sacc}E_t$ pH-rate Profile.

Enzyme	pK_1	pK_2	pH Independent Value (M^1s^{-1})	Fold Decrease
WT	7.6 ± 0.1	9.9 ± 0.2	$(2.9 \pm 0.5) \times 10^4$	
D126A	8.8 ± 0.3	9.9 ± 0.3	$(1.0 \pm 0.3) \times 10^4$	2.9
C154S	8.1 ± 0.2	10.3 ± 0.2	74 ± 8	391
Y99F	7.1 ± 0.4	9.5 ± 0.3	11 ± 2	2536
D126A-Y99F	9.3 ± 0.2	10.3 ± 0.5	86 ± 22	337
D126A-C154S	8.5 ± 0.2	ND ^a	56 ± 8	518

^aND is not determined.

Table 5Solvent Kinetic Isotope Effects.^a

Enzyme	D ₂ O V_2	D ₂ O (V_2/K_{Sacc})
WT ^b	1.8 ± 0.1	1.8 ± 0.1
D126A	2.6 ± 0.2	1.9 ± 0.6
C154S	2.8 ± 0.7	2.8 ± 0.7
Y99F	2.8 ± 0.1	1.9 ± 0.2
D126A-C154S	2.6 ± 0.1	2.22 ± 0.03
D126A-Y99F	2.1 ± 0.1	3.1 ± 0.3

^a Data were obtained in the pH(D) independent region of the pH rate profiles for V_2 and V_2/K_{Sacc} for each of the mutant enzymes.^b From ref. (6).