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Determinants of Substrate Specificity for Saccharopine Dehydrogenase from *Saccharomyces cerevisiae*†

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Abstract

A survey of NADH, α-Kg, and lysine analogues has been undertaken to define the substrate specificity of saccharopine dehydrogenase, and to identify functional groups on all substrates and dinucleotides important for substrate binding. A number of NAD analogues, including NADP, 3-acetylpyridine adenine dinucleotide (3-APAD), 3-pyridinealdehyde adenine dinucleotide (3-PAAD), and thionicotinamide adenine dinucleotide (thio-NAD), can serve as a substrate in the oxidative deamination reaction, as can a number of α-keto analogues, including glyoxylate, pyruvate, α-ketobutyrate, α-ketovalerate, α-ketomalonate, and α-ketoadipate. Inhibition studies using nucleotide analogues suggest that the majority of the binding energy of the dinucleotides comes from the AMP portion, and that distinctly different conformations are generated upon binding of the oxidized and reduced dinucleotides. Addition of the 2'-phosphate as in NADPH causes poor binding of subsequent substrates, but has little effect on coenzyme binding and catalysis. In addition, the 10-fold decrease in affinity of 3-APAD in comparison to NAD suggests that the nicotinamide ring binding pocket is hydrophilic. Extensive inhibition studies using aliphatic and aromatic keto acid analogues have been carried out to gain insight into the keto acid binding pocket. Data suggest that a side chain with 3 carbons (from the α-keto group up to and including the side chain carboxylate) is optimal. In addition, the distance between the C1-C2 unit and the C5 carboxylate of the α-keto acid is also important for binding; the α-oxo group contributes a factor of 10 in affinity. The keto acid binding pocket is relatively large and flexible, can accommodate the bulky aromatic ring of a pyridine dicarboxylic acid, and a negative charge at the C3 but not the C4 position. However, the amino acid binding site is hydrophobic and the optimal length of the hydrophobic portion of amino acid carbon side chain is 3 or 4 carbons. In addition, the amino acid binding pocket can accommodate a branch at the γ-carbon, but not at the β-carbon.

Saccharopine dehydrogenase (*N*6-(glutaryl-2)-L-lysine: NAD oxidoreductase (L-lysine forming); (EC 1.5.1.7)) (SDH[1]) catalyzes the last step of the α-aminoadipate (AAA) pathway for the *de novo* synthesis of L-lysine in fungi. The reaction involves the reversible pyridine

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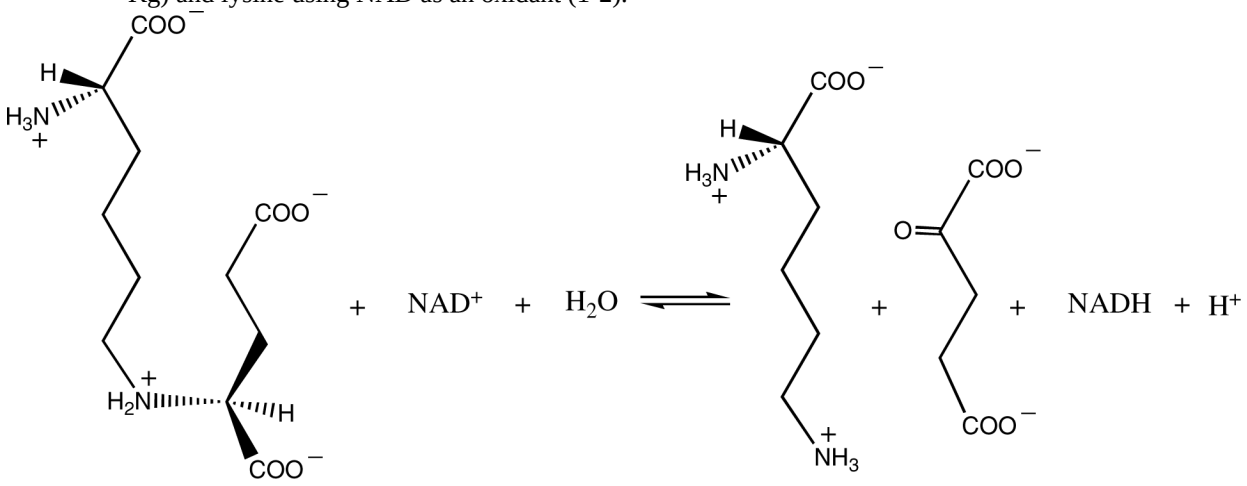
[1]Abbreviations: SDH, saccharopine dehydrogenase; AAA, α-aminoadipate pathway; NAD(P), β-nicotinamide adenine dinucleotide (phosphate) (the + charge is omitted for convenience); NADH(P), reduced β-nicotinamide adenine dinucleotide (phosphate); NADD, reduced nicotinamide adenine dinucleotide with deuterium in the 4*R* position; AMP, adenosine 5'-monophosphate; ADP, adenosine 5'-diphosphate; NMN, nicotinamide mononucleotide; 3-PAAD, 3-pyridinealdehyde adenine dinucleotide; 3-APAD, 3-acetylpyridine adenine dinucleotide; thio-NAD, thio-nicotinamide adenine dinucleotide; 2',3'-cyclic NADP, β-nicotinamide adenine dinucleotide 2',3'-cyclic monophosphate; α-Kg, α-ketoglutarate; α-Kb, α-ketobutyrate; α-Kv, α-ketovalerate; α-Kiv, α-ketoisovalerate; α-Km, α-ketomalonate; α-Ka, α-ketoadipate; α-Kp, α-ketopimelate; OAA, oxaloacetate; OG, oxalylglycine; C, competitive; UC, uncompetitive; NC, noncompetitive; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'- (2-ethanesulfonic acid); Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Taps, *N*-[tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; DCl, deuterium chloride; NaOD, sodium deuteroxide; D_2O, deuterium oxide.

nucleotide-dependent oxidative deamination of saccharopine to generate α-ketoglutarate (α-Kg) and lysine using NAD as an oxidant (1-2).



The histidine-tagged SDH from *Saccharomyces cerevisiae* has been overexpressed in *Escherichia coli* and purified to ~98% using Ni-NTA chromatography (3). The SDH reaction proceeds via an ordered kinetic mechanism in which NAD is the first substrate bound followed by saccharopine in the physiologic reaction direction, while in the reverse reaction direction, NADH adds to enzyme first, and there is no preference for binding of α-Kg and lysine at pH 7.0. A general mechanism for the oxidative deamination of saccharopine has been proposed on the basis of pH-rate profiles and isotope effects, Scheme 1 (4). A group on the enzyme with a pK_a of 6.2 is required to deprotonate the secondary amine of saccharopine as it is oxidized to an imine intermediate, and this group ultimately functions to donate a proton to the ε-amine of lysine as it is released as a product. A second enzyme group with a pK_a of 7.2 acts to activate water in the imine hydrolysis step and then shuttles protons between itself and reaction intermediates giving the final lysine and α-Kg products. A concerted hydride and proton transfer to oxidize saccharopine to an imine and subsequent formation of a carbinolamine intermediate as the imine is hydrolyzed contribute to rate limitation.

Saccharopine dehydrogenase from *S. cerevisiae* shows a high degree of coenzyme specificity (5,6). NADPH is a poor substrate, and causes an increase in the K_m values for α-Kg and lysine, while it was reported that NADP is not a substrate in the direction of saccharopine oxidation (6). The dehydrogenase also exhibits stringent substrate specificity with respect to its keto acid substrate (5,7). Only pyruvate was shown to replace α-Kg as an alternative substrate giving ε-N-(L-propionyl-2)-L-lysine (8). In addition, the enzyme shows an absolute specificity toward its amino acid substrate L-lysine. Inhibition studies by lysine substrate analogues suggest that a hydrophobic interaction between the side chain of an amino acid and the enzyme is important in binding the amino acid reactant (7).

As a result of studies of the kinetic and chemical mechanism of SDH (3,4), and a consideration of previously published data (5-8), a number of questions were raised. For example, it is extremely unlikely that NADPH is a substrate but NADP is not. The specificity determinants for binding saccharopine are not clear, nor are those for substrate specificity in either the oxidative deamination or reductive amination reaction directions. In this study, we show that a number of NAD analogues, including NADP, can serve as a substrate in the oxidative deamination reaction, as can a number of α-keto acid analogues in the opposite reaction direction; the enzyme is specific for lysine. In addition, a survey of inhibitory substrate analogues has been carried out to map the specificity of the active site of SDH.

MATERIALS AND METHODS

Chemicals

L-Saccharopine, L-lysine, D-lysine, L-ornithine, L-valine, L-norvaline, L-methionine, L-leucine, L-isoleucine, L-asparagine, L-glutamine, L-arginine, adenosine, AMP, ADP, ADP-ribose, NMN, 2',3'-cyclic NADP, 3-APAD, 3-PAAD, thio-NAD, α-ketoglutarate (α-Kg), pyruvate, glyoxylate, α-ketobutyrate (α-Kb) , α-ketovalerate (α-Kv), α-ketomalonate (α-Km), α-ketoadipate (α-Ka), malonate, oxaloacetate (OAA), glutarate, adipate, α-ketoisovalerate (α-Kiv), α-ketopimalate (α-Kp), pyridine 2,4-dicarboxylate, pyridine 2,3-dicarboxylate, pyridine 2,5-dicarboxylate, L-pipecolic acid, yeast lactate, alcohol, and aldehyde dehydrogenases were obtained from Sigma. β-NAD(P)H and β-NAD(P) were purchased from USB. Oxalylglycine (OG) was from Frontier Scientific. Oxalate and succinate were from Fisher Scientific. Mes, Hepes, Ches, and Taps were from Research Organics, Inc. Deuterium oxide (D_2O) (99 atom % D) and ethanol-d_6 (99 atom % D) were purchased from Cambridge Isotope Laboratories, Inc. AG MP-1 and Bio-Gel P-2 resins were from Bio-Rad. All other chemicals and reagents were obtained from commercial sources, were reagent grade, and were used without further purification.

Synthesis of 4R-NADD

The 4*R*-NADD was prepared according to (4). The reaction mixture, contained ethanol-d_6, NAD, yeast alcohol and aldehyde dehydrogenases, and was titrated to 9.0 with KOH throughout the reaction. Once the reaction was quenched with several drops of CH_3Cl, the aqueous layer was subjected to ion exchange chromatography on a AG MP-1 column (4 × 25 cm). The chromatogram was developed with 1 M LiCl, pH 10.0, and the 4*R*-NADD was then desalted via a Bio-Gel P-2 column (1.6 × 60 cm). Concentrations of the NADD stock solutions were estimated spectrophotometrically using a ε_{340} of 6.22 $mM^{-1}cm^{-1}$.

Cell growth, SDH expression and purification were carried out as reported previously (3). Glycerol (50%) was added to the stock enzyme solution to minimize any activity loss, and the diluted enzyme solution used for activity studies was prepared fresh daily.

Enzyme Assays

The SDH-catalyzed oxidative deamination reaction was followed by monitoring the increase (or decrease) in absorbance at 340 nm as NAD(P) is reduced or NAD(P)H is oxidized, and by monitoring the increase in absorbance at 363, 358, and 395 nm as 3-APAD, 3-PAAD, and thio-NAD are reduced, respectively. The following extinction coefficients were used for NAD(P)H, reduced 3-APAD, 3-PAAD, and thio-NAD: 6.22 $mM^{-1}cm^{-1}$, 9.1 $mM^{-1}cm^{-1}$, 9.3 $mM^{-1}cm^{-1}$, and 11.3 $mM^{-1}cm^{-1}$ (9). Reactions were carried out in semi-micro quartz cuvettes with a path length of 1 cm in a final volume of 1 or 0.5 ml containing 100 mM buffer (Mes, pH 5.5−6.8; Hepes, pH 6.8−8.2; Ches, pH 8.2−10.0), and variable concentrations of substrates as indicated below. Initial velocities were measured with a Beckman DU640 UV/Vis spectrophotometer. All kinetic assays were performed at 25 °C and the temperature was maintained using a Neslab RTE-111 circulating water bath. A unit of enzyme activity is defined as the amount of enzyme catalyzing the production or utilization of 1 μmole of dinucleotide substrate (or analogue) per min at 25°C. Reactions were initiated by the addition of a small amount (10 or 20 μL) of appropriately diluted enzyme solution to a mixture that contained all other reaction components, and the initial linear portion of the time course was used to calculate the initial velocity.

Systematic Initial Velocity Studies

When NADPH was used as the coenzyme in the direction of saccharopine formation, a systematic initial velocity study was performed at pH 7.0 according to (3). Briefly, the initial rate was measured as a function of the NADPH concentration and at different fixed levels of α-Kg and a fixed concentration of lysine. The experiment was then repeated at different lysine concentrations. The concentration ranges for NADPH, α-Kg, and lysine are 0.05−0.3, 1−10, and 2−20 mM, respectively. Systematic initial velocity studies were also carried out when pyruvate served as the keto acid substrate. In this case, initial rates were measured as a function of the pyruvate concentration (5−50 mM) at different fixed levels of lysine (30−300 mM), with NADH fixed at 0.2 mM at pH 7.0. The amount of enzyme used for each reaction was 6.5 times that used in SDH reactions with the natural substrates (3).

In the direction of saccharopine oxidation, initial rates were measured as a function of the concentration of the coenzyme analogues, NADP, 3-APAD, 3 -PAAD, and thio-NAD, respectively, with saccharopine fixed at 6 mM; the pH was maintained at 7.55 or 9.2. Since the concentration of saccharopine used was relatively low (~K_m), K_{ia} values of oxidized dinucleotides were obtained; these values are dissociation constants for E-dinucleotide in an ordered mechanism. In order to estimate the apparent K_m values of the keto acid substrates, glyoxylate, α-Kb, α-Kv, α-Km, and α-Ka, initial rates were measured as a function of the concentration of keto acid substrates, with NADH fixed at 0.2 mM, and lysine at 20 or 40 mM, at pH 7.0.

Product and Dead-end Inhibition Studies

Inhibition patterns were obtained by measuring the initial rate at different concentrations of one reactant, with the concentrations of the other reactants fixed at their K_m values, and at different fixed concentrations of the inhibitor including zero. In most cases, an initial estimate of the K_i for the inhibitor was obtained by fixing the varied substrate at its K_m value and varying the inhibitor concentration. The appK_i is estimated by Dixon analysis, a plot of 1/*v* vs. **I**, extrapolating to 1/*v* equal to zero, and dividing by 2.

Double Inhibition Studies

Double-inhibition studies were performed to determine whether synergism of binding occurs between analogues of α-Kg and lysine. In the direction of saccharopine formation, initial rates were measured as a function of OG (0−0.2 mM) or pyridine 2,4-dicarboxylic acid (0−4 mM), respectively, at different fixed levels of ornithine (0−15 mM), with the concentration of NADH maintained saturating (10K_m), while α-Kg and lysine were maintained at their respective K_m values.

pK_i Profile of AMP

Data were obtained according to (4). The inhibition constant of AMP was obtained as a function of pH by measuring the initial rate as a function of NADH with α-Kg and lysine fixed at their K_m values. The experiment was then repeated at different fixed levels of AMP including zero. Data were obtained with 100 mM Mes 5.5−6.8, Hepes, 6.8−8.2, and Ches; 8.2−10.0 used to maintain the pH. Sufficient overlap was obtained upon changing buffers to determine whether the buffer effects the reaction; no effects were detected. The enzyme is stable when incubated for 20 min over the pH range 5.5−9.8. The pK_i profile was then evaluated graphically for quality of data by plotting log(1/K_i) against pH.

Product Identification by NMR

When keto acid analogues were used as slow substrates, products were identified by measuring, at 21°C, the ^{1}H NMR chemical shifts of protons β to the secondary amine nitrogen of the new

products. Reaction mixtures containing 2 mM NADH, 20 mM keto acid analogues, and 2 mM lysine were prepared in 20 mM K_2HPO_4 buffer, and were adjusted to a pD value of 7.4 using either DCl or NaOD before SDH was added. The purified SDH was dialyzed against 20 mM K_2HPO_4 at pD 7.4. Once the enzyme was added, the reaction mixtures were kept at 4°C overnight, allowing sufficient time for the reaction to reach completion (monitored spectrophotometrically). Once the reactions were complete, several drops of $CHCl_3$ were added to the mixtures to precipitate the enzyme, and sufficient heat acid-activated charcoal was then added to remove the dinucleotides. The remaining reaction mixture was then analyzed by ^{1}H NMR.

All NMR experiments were performed on a Varian Mercury VX-300 MHz spectrometer with a Varian 4-nuclei auto-switchable PFG probe. ^{1}H NMR spectra were collected using the PRESAT pulse sequence supplied by Varian, Inc. The spectra were collected with a sweep width of 4803.1 Hz, 8 transients, and an acquisition time of 1.998 seconds.

Deuterium Kinetic Isotope Effects

Primary deuterium kinetic isotope effects were measured for the reaction in which pyruvate was substituted for α-Kg by direct comparison of initial rates with NADH and NADD. Initial rates were measured as a function of one substrate concentration at fixed concentrations of the other two. The fixed concentrations used for NADH(D), pyruvate, and lysine are 0.2 mM (~3K_m), 50 mM (~12.5K_m), and 300 mM (~5K_m), respectively.

Isotope effects are expressed using the nomenclature developed by Northrop (10) and Cook and Cleland (11). Primary deuterium kinetic isotope effects are written with a leading superscript D, e.g. a primary deuterium isotope effect on V/K is written $^D(V/K)$.

Data Processing

Initial velocity patterns were plotted in double reciprocal fashion to assess data quality and determine the appropriate equation for fitting. Data were then fitted to appropriate equations as discussed below, using the Marquardt-Levenberg algorithm 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 systematic initial velocity studies using α-Kg substrate analogues in the direction of saccharopine formation, or using NADPH as the coenzyme substrate were fitted using eq 1 for a terreactant kinetic mechanism (3). When NADP, 3-APAD, 3-PAAD, and thio-NAD were used as coenzyme, respectively, in the direction of saccharopine oxidation, data were fitted using eq 2. When concentrations of pyruvate and lysine were varied at a fixed NADH concentration, data obtained were fitted using eq 3 for a bireactant mechanism. Competitive inhibition patterns were fitted using eq 4, while uncompetitive inhibition patterns were fitted using eq 5. Data for double inhibition studies by OG/ornithine and pyridine 2,4-dicarboxylic acid/ornithine were fitted using eq 6. Data for V and V/K deuterium isotope effects were fitted using eq 7, and data for the AMP pK_i profile were fitted manually using eq 8.

$$v=\frac{V\mathbf{ABC}}{K_{ia}K_{ib}K_c+K_{ib}K_c\mathbf{A}+K_{ic}K_a\mathbf{B}+K_{ia}K_b\mathbf{C}+K_c\mathbf{AB}+K_b\mathbf{AC}+K_a\mathbf{BC}+\mathbf{ABC}} \quad (1)$$

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

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

$$v=\frac{V\mathbf{A}}{K_a\left(1+\frac{\mathbf{I}}{K_{is}}\right)+\mathbf{A}} \quad (4)$$

$$v=\frac{V\mathbf{A}}{K_a+\mathbf{A}\left(1+\frac{\mathbf{I}}{K_{ii}}\right)} \quad (5)$$

$$v=\frac{v_o}{1+\frac{\mathbf{I}}{K_i}+\frac{\mathbf{J}}{K_j}+\frac{\mathbf{IJ}}{\alpha K_i K_j}} \quad (6)$$

$$v=\frac{V\mathbf{A}}{K_a\left(1+F_i E_{V/K}\right)+\mathbf{A}\left(1+F_i E_V\right)} \quad (7)$$

$$\log \quad y=\log\frac{\left[\frac{Y_H}{\left(1+\frac{K_2}{H}\right)}+Y_L\left(\frac{H}{K_1}\right)^2\right]}{\left[1+\left(\frac{H}{K_1}\right)^2\right]} \quad (8)$$

In eqs 1-7, v and V are initial and maximum velocities, **A**, **B**, and **C** are substrate concentrations, **I** is inhibitor concentration, K_a, K_b, and K_c are Michaelis constants for substrates A, B, and C, respectively, while K_{ia}, K_{ib}, and K_{ic} are defined as inhibition constants for A from EA, B from EAB, and C from EAC; these constants are also dissociation constants for the respective complexes. In eqs 4-5, K_{is} and K_{ii} represent inhibition constants for the slope and intercept, respectively; the values obtained from competitive inhibition are dissociation constants for the EI complex. In eq. 6, K_i and K_j are dissociation constants for EI and EJ complexes, respectively, v_0 is the rate in the absence of inhibitors, and α is the interaction constant that estimates the influence of one inhibitor upon binding of the other. In eq 7, F_i is the fraction of deuterium label in the substrate, and $E_{V/K}$ and E_V are the isotope effects minus 1 on V/K and V, respectively. In eq 8, y is the observed value of the parameter ($1/K_i$) as a function of pH, H is the hydrogen ion concentration, K_1 and K_2 represent acid dissociation constants for enzyme or substrate functional groups important in a given protonation state for optimal binding and/or catalysis, and Y_L and Y_H are constant values of $1/K_i$ at low and high pH, respectively.

RESULTS

Initial Velocity Studies with NAD/NADH Analogues

In the physiological reaction direction, lysine formation, NADP, 3-APAD, 3-PAAD, and thio-NAD all can serve as the oxidant in the oxidative deamination reaction, Figure 1, while in the reverse reaction direction, NADPH can substitute for NADH. In the direction of saccharopine oxidation, initial rates were obtained as a function of the concentrations of NAD analogues and fixed concentration of saccharopine (6 mM) at pH 7.55 and 9.2. Estimates of the kinetic parameters are provided in Table 1.

Double-reciprocal initial velocity patterns in the direction of reductive amination using NADPH as the reductant were obtained by varying the concentrations of NADPH and α-Kg at different fixed concentrations of lysine at pH 7.0 and 25 °C. The crossover points for all double-reciprocal plots are to the left of the ordinate (data not shown). All of the initial velocity data were fitted to eq 1 for a terreactant mechanism. Substrate inhibition by α-Kg and lysine was observed as α-Kg and lysine concentration increased above 50 mM and 100 mM, respectively (data not shown). Kinetic parameters are summarized in Table 2 and compared to data obtained previously with NADH (3).

Inhibition Studies to Determine the Kinetic Mechanism with NADP

In the direction of saccharopine formation, product inhibition by NAD is competitive vs NADH. Similarly, product inhibition by NADP is competitive vs NADPH when NADPH serves as the coenzyme, while dead-end inhibition by leucine (lysine analogue) is uncompetitive vs NADPH.

Initial Velocity Studies with Keto Acid Substrate Analogues

In addition to pyruvate, five keto acids have been identified as alternative substrates for the SDH-catalyzed reaction in the direction of saccharopine formation, Figure 2A. These include glyoxylate, α-Kb, α-Kv, α-Km, and α-Ka. Kinetic parameters obtained by measuring the initial rate at fixed concentrations of NADH and lysine are summarized in Table 3.

Initial velocity patterns obtained with pyruvate as the substrate, and varying the concentrations of pyruvate and lysine with NADH fixed at 0.2 mM (~3K_m) intersects to the left of the ordinate (data not shown), consistent with the proposed sequential kinetic mechanism (3). All kinetic parameters are listed in Table 3.

With glyoxylate as the substrate, a background rate was observed when the absorbance at 340 nm was monitored in the absence of enzyme. The stock glyoxylate solution (500 mM) has a slight yellow color, and this was thought to account for the absorbance at 340 nm. The increased presence of D_2O decreased the background rate, suggesting it is related to the dehydration of glyoxylate. A solvent deuterium isotope effect has been observed for dehydration of glyoxylate (12). Thus, the SDH reaction in the presence of glyoxylate was corrected for the background rate. Kinetic parameters are shown in Table 3.

Although there appeared to be an OAA-dependent decrease in A_{340} in the presence of NADH, lysine, and SDH, the rate was due to pyruvate contaminating the OAA solution, a result of nonenzymatic decarboxylation, as shown using lactate dehydrogenase.

Confirmation of Product of Reaction with Alternative α-Keto Acid Substrates

When keto acid analogues were used as alternative substrates, products were identified by measuring the ^{1}H NMR chemical shifts of protons β to the secondary amine nitrogen in the products. A control spectrum with lysine exhibits chemical shifts at 3.7 and 3.0 ppm, close to product peaks at 3.5 and 2.9 ppm, corresponding to C(8)-H and C(6)-H_2 of saccharopine, respectively (4). However, there is no overlap of the chemical shifts of the keto acid analogues and products. Therefore, experiments were carried out with the keto acid analogue in excess and with lysine limiting. Completeness of the reaction was monitored using the absorbance at 340 nm in a separate reaction using similar conditions. In all cases the chemical shifts corresponding to the product C(8)-H and C(6)-H_2 resonances were observed, confirming product formation.

Deuterium Kinetic Isotope Effects

Primary deuterium kinetic isotope effects with pyruvate in place of α-Kg were measured by direct comparison of initial rates at pH 7.0 with A-side NADH(D). Finite effects were observed on all kinetic parameters, and are listed in Table 4.

Dead-end Inhibition by NAD/NADH Analogues

Inhibition by dinucleotide analogues AMP, ADP, ADP-ribose, NMN, 2',3'-cyclic NADP, and NADP, was competitive vs NADH at pH 7.0. All inhibition constants are summarized in Table 5 and structures are provided in Figure 1.

pH Dependence of the K_i for AMP

The pH dependence of the dissociation constant of AMP was determined in order to obtain an estimate of intrinsic p*K* value(s) of group(s) required for optimum binding of NADH. Over the pH range studied, AMP is a competitive inhibitor against NADH, and the pK_{iAMP} profile is shown in Fig. 3. The pK_i decreases from a constant value at pH 7.0 to another constant value below a pH of 6.0, and decreases as pH increases above 7.5. The decrease at low pH exhibits a slope of 2 indicating two groups must be unprotonated for optimum binding of AMP, while the decrease at high pH is with a slope of −1 indicating a group must be protonated for optimum binding of AMP. An average pK_a value of about 7.1 is estimated for all three of the groups.

Dead-end Inhibition by Keto Acid Analogues

A number of α-Kg analogues exhibit competitive inhibition patterns against α-Kg at pH 7.0 in the direction of saccharopine formation, Figure 2B and Table 6. In addition, pyridine 2,5-dicarboxylate and L-pipecolic acid gave no observable inhibition at a concentration of 50 mM.

Dead-end Inhibition by Amino Acid Analogues

SDH shows very strict substrate specificity towards its amino acid substrate, L-lysine. A number of lysine analogues show competitive inhibition vs L-lysine, including L-ornithine and D-lysine, Figure 4. L-Asparagine gave no inhibition at a concentration of 50 mM. Since L-leucine, a competitive inhibitor of L-lysine, binds better at pH values below 6, the K_i for amino acid analogues with aliphatic side chains such as L-valine and L-isoleucine were measured at pH 6.0 and 7.0. All K_i values are summarized in Table 7.

Double Inhibition Studies

In order to determine whether the binding of lysine and α-Kg affect one another, double-inhibition experiments with competitive inhibitors of the two substrates were carried out. The initial rate was measured at saturating NADH, with α-Kg and lysine fixed at their respective K_m values, and varying the concentrations of OG and ornithine, Fig. 5. The rate in the absence of inhibitors, v_0, was 11.8 ± 0.2 μM/min, while the appK_i values for E-NADH-OG and E-NADH-ornithine complexes are 0.050 ± 0.002 and 4.6 ± 0.2 mM, respectively. The interaction constant, α, was 0.7 ± 0.1, indicating slight synergism of binding between OG and ornithine. A double-inhibition pattern obtained with ornithine and the bulky pyridine 2,4-dicarboxylic acid gave quite different results, with K_i values for E-NADH-pyridine 2,4-dicarboxylic acid and E-NADH-ornithine complexes of 1.99 ± 0.06 and 5.0 ± 0.1 mM, respectively. A value of 2.4 ± 0.4 for was estimated indicating antagonism of binding.

DISCUSSION

The substrate specificity exhibited by most enzymes is a hallmark of biological systems, which distinguishes them from non-biological catalysts. A survey of substrate analogues was undertaken to determine which substrate functional groups are responsible for substrate binding, to determine the stereochemistry involved in substrate/inhibitor binding, and to potentially define the geometry of the substrate binding site. These aspects are discussed below.

NAD/NADH Analogues

Kinetic Mechanism with NADP/NADPH—In contrast to previously published results (6), NADP does serve as a substrate, albeit poorly, in the direction of saccharopine oxidation. Under the conditions we examined, 6.5 times more enzyme was needed with NADP compared to assay conditions with NAD as the natural substrate at pH 7.55 and 9.2 (3). The K_{ia} value of NADP increased about 2-fold, which is the same extent as the increase in the K_{ia} of NADPH

comparing to that of NADH, suggesting that the presence of the phosphate at the C2 of the adenosine moiety of the coenzyme decreases its affinity only slightly.

In the direction of saccharopine formation, substitution of NADPH for NADH results in the formation of saccharopine. Systematic initial velocity studies were carried out by varying the concentrations of NADPH and α-Kg at different fixed concentrations of lysine at pH 7.0 and 25°C. All of the double-reciprocal plots intersect to the left of the ordinate, indicating a sequential kinetic mechanism. When data obtained were fitted to eq 1 for the terreactant mechanism defined using NADH as a substrate (3), all terms in the denominator of the rate equation were present, and all parameters were well defined, consistent with a similar mechanism with NADPH as the substrate. To further test this hypothesis, inhibition studies were carried out and are discussed below.

As a competitive inhibitor of lysine, leucine competes with lysine for its binding site on the enzyme (3). An uncompetitive inhibition pattern is observed vs NADPH, indicating that leucine or lysine binds to an enzyme form that exists when NADPH is saturating, suggesting that NADPH is the first substrate bound to the free enzyme in a sequential kinetic mechanism. The order of product release in the direction of saccharopine formation was examined using product inhibition by NADP. The competitive inhibition by NADP vs NADPH at low α-Kg and lysine concentrations indicates that NADP binds to the free enzyme, competing with NADPH, suggesting it is the last product released in the direction of saccharopine formation.

The K_i value for NADPH increased about 2-fold compared to that of NADH. However, the presence of the 2'-phosphate resulted in about an order of magnitude increase in the Michaelis constants for all of the substrates. K_m is equal to the product of the steady state concentration of substrate and the enzyme forms that are present at near zero substrate concentration divided by the concentration of enzyme forms that are present at saturating substrate concentration. Thus, the increase in K_m likely reflects either an increase in the off-rate for NADP, which would increase the concentration of enzyme forms at near zero substrate, or a decrease in the affinity of the E-NADPH complex for lysine and α-Kg. Given the data in Table 2, it is the latter that is affected; note the 10-fold increase in $K_{i\alpha\text{-}Kg}$ when NADPH is the substrate compared to that with NADH. Data are consistent with a difference in the conformation of the E-NADH and E-NADPH complexes. In agreement, K_{iLeu} also increases by a factor of 10 as the cofactor changes from NADH to NADPH.

When initial rates were measured with NADPH as the coenzyme, the double-reciprocal plot exhibited substrate inhibition as the concentrations of α-Kg and lysine were increased above 50 mM and 100 mM, respectively. The observed substrate inhibition by α-Kg suggests the existence of the dead-end E-NADP-α-Kg complex, as is true with NADH as the substrate (3). In order to form this complex, the E-NADP form must exist in the steady state, which suggests that the release of NADP from E-NADP also contributes to the overall rate limitation. The substrate inhibition by lysine suggests the existence of the dead-end E-Lys complex at high lysine concentrations, also observed with NADH as the substrate (3).

Substrate Analogues of NAD—In addition to NADP, the dinucleotide analogues 3-APAD, 3-PAAD, and thio-NAD that contain modified nicotinamide rings, all support the oxidative deamination reaction. Because the concentrations of saccharopine used were roughly estimated to be equal to K_m, K_{ia} values, dissociation constants for each of the dinucleotide analogues from the E-dinucleotide complex, and apparent V_{max} values were obtained. The K_{ia} values obtained at pH 7.55 and 9.2 indicate that there is a slight, if any, pH dependence over this range, as is true for NAD (4). 3-PAAD and thio-NAD bind to free enzyme with about the same affinity as NAD, suggesting that the amide NH_2 of the nicotinamide ring does not contribute much in terms of coenzyme binding. The K_{ia} value for 3-APAD is increased 10-

fold, suggesting that the binding pocket of the nicotinamide ring is relatively hydrophilic and cannot accommodate the hydrophobic methyl group of 3-APAD very well.

Inhibitory Nucleotide Analogues—K_i values for dead-end inhibitors provide information on the importance of substrate binding since they occupy the same binding site as the substrate. With NAD(P)H as the varied substrate, NAD, NADP and 2',3'-cyclic NADP all have about the same K_i, suggesting little or no effect of the 2'-phosphate on dinucleotide binding. NMN binds poorly to the dinucletotide-binding site, with a 5-fold lower affinity. Some of the lower affinity can be explained by the 75-fold tighter binding of NADH than NAD, and the tighter binding of NAD at pH values above 7.2 (3). Consistent with this suggestion, ADP-ribose binds to free enzyme with a 5-fold increased affinity. This is still about 20-fold lower than the affinity for NADH, indicative of the preference for the reduced nicotinamide ring. Elimination of the nicotinamide portion of the molecule as in ADP gives a 3-fold increase in affinity compared to ADP-ribose, but still 5-fold lower than NADH; while AMP has only a 3-fold lower affinity compared to NADH. Nonetheless, AMP binds 30-fold tighter than NAD. Adenosine binds with a 8-fold lower affinity compared to AMP, indicative of the importance of the α-phosphate.

Data suggest that the majority of the binding energy of NAD comes from the AMP portion. In the case of ADP-ribose, the β-phosphate groups account for the lower affinity, suggesting that the binding pocket is slightly hydrophobic and/or negatively charged. Since NAD binds with about 2-orders of magnitude lower affinity compared to NADH, data suggest distinctly different conformations generated upon binding the two dinucleotides.

pH Dependence of the K_i for AMP—The pK_i profile for AMP, a competitive inhibitor of NADH, indicates that two groups with pK_a values of about 7.1 must be unprotonated, and one group with about the same apparent pK_a must be protonated for optimal binding of AMP. Since AMP has only one pK_a over the pH range 5−10, a value of 6.2−6.4 for the 5'-phosphate, one of the groups with a pK_a of 7.0 that must be unprotonated and the group with a pK_a of 7.2 that must be protonated must come from the enzyme. On the basis of the proposed chemical mechanism of SDH, the two groups seen in the pK_{iAMP} profile are most likely Asp 281 and Lys 77 (4). The former, with a pK_a of about 6.2, is important for binding of saccharopine and likely serves as a general base in the oxidation step, while the group with a pK_a of about 7.2, is likely responsible for activating water in hydrolysis of the imine formed upon oxidation of saccharopine, and is known to affect the binding of NAD.

Dinucleotide Specificity for Amino Acid Dehydrogenases—Saccharopine dehydrogenase (SDH) belongs to the class of pyridine nucleotide-linked amino acid oxidoreductases. Enzymes in this class include dehydrogenases that catalyze the oxidative deamination of amino acids such as L-glutamate (GluDH), L-alanine (AlaDH), L-leucine (LeuDH), and L-phenylalanine (PheDH) dehydrogenases. The majority of the enzymes are *pro-S* specific for hydride transfer, but SDH, like AlaDH and L-lysine ε-dehydrogenase, is *pro-R* specific.

An interesting aspect of coenzyme specificity within the group is their discrimination between NAD(H) and NADP(H), i.e., for or against the presence of a 2'-phosphate group. In this regard, GluDH may have a preference for NAD, NADP, or both dependent on cellular location and function (14). The bacterial L-lysine ε-dehydrogenase is specific for NAD (15), while the yeast enzyme is NADP-specific (16). All known examples of LeuDH (17) are specific for NAD, as are PheDH (18) and AlaDH (19).

Many of the dehydrogenases have been studied with respect to alternative dinucleotide substrates (15-19). 3-APAD is a better substrate for LeuDH (166%) and PheDH (241%) than is NAD, and thio-NAD (101%) is as good as NAD for PheDH. However, 3-PAAD (3−20%)

is a poor substrate for all of the amino acid dehydrogenases, and all three of the alternative diuncleotide substrates are poor substrates for LysDH (3-APAD, 16%; 3-PAAD, 0.3%; thio-NAD, 0.2%). In the case of SDH, all of the alternative dinucleotide substrates are poor substrates, but as for the other enzymes studied 3-APAD is the best by at least a factor of 5−6.

It is interesting to note that no studies of synergism of substrate binding have been carried out. Double inhibition experiments are relatively easy to perform and can provide important information on binding of substrates in sequential kinetic mechanism. Hopefully this technique will be utilized more frequently in the future.

Keto Acid Analogues

Kinetic Mechanism with Pyruvate—The kinetic mechanism of SDH with pyruvate as the keto acid substrate has been fully characterized by initial velocity, dead-end inhibition, and primary deuterium kinetic isotope effect studies. The Initial velocity pattern obtained by varying the concentrations of pyruvate and lysine at a fixed level of NADH intersects to the left of the ordinate (data not shown), suggesting a sequential mechanism. Uncompetitive inhibition by leucine vs NADH indicates that NADH is the first substrate bound. Data suggest that the kinetic mechanism with pyruvate is the same as that observed with the natural substrate, α-Kg.

When both NADH and NADD were used for the pyruvate reaction, finite isotope effects were observed for all kinetic parameters. Given that $^{D}(V/K_{NADH})$, $^{D}(V/K_{pyruvate})$, and $^{D}(V/K_{Lys})$ are identical, within error, and initial velocity data suggest ordered addition of NADH before lysine and pyruvate, data suggest an equilibrium mechanism. However, the isotope effect on V is smaller with pyruvate as the substrate compared to the α-Kg reaction, suggesting that release of the product NAD still contributes to rate-limitation.

Substrate Analogues of Keto Acid—Several α-keto acids can replace α-Kg, including glyoxylate, α-Kb, α-Kv, α-Km, and α-Ka, Table 3. The V/KE_t values for all of the alternative substrates relative to α-Kg are decreased by 300- (α-Ka) to 2700- (pyruvate) fold, suggesting that the γ-carboxylate of α-Kg is important for proper binding of the α-keto acid substrate. However, OAA is not a substrate, and α-Ka is a substrate, but with a 5-fold lower V/E_t value, indicating that the position of the side chain carboxylate is important. The V/E_t for α-Kb is identical to that with α-Kg, while that of α-Kv is decreased by only about 2-fold. Data suggest that the optimum length of the side chain is 3 carbons (from the α-keto group up to and including the side chain carboxylate). In addition, α-Km can serve as a substrate but not OAA, suggesting that the binding pocket of the keto acid can accommodate a negative charge at the C3 but not the C4 position.

Inhibitory Keto Acid Analogues—The specificity of the keto acid substrate-binding pocket was assessed using mimics of α-Kg. Two classes of inhibitors, aliphatic and aromatic, were chosen as probes of the α-Kg binding site of SDH. With the exception of pyridine 2,5-dicarboxylate and L-pipecolic acid, which exhibit no inhibition at high concentrations, and OAA and glutarate, which exhibit noncompetitive inhibition, all other compounds are competitive inhibitor of α-Kg. Glutarate and OAA give noncompetitive inhibition as a result of combination with E-NADH and E-NADH-Lys complexes (4). From these data, Table 6, the keto acid binding site is assessed with respect to the spatial relationship and distance between the C1-C2 unit and the C5 carboxylate.

Oxalylglycine, the tightest binding inhibitor discovered to date, is structurally most similar to α-Kg, and suggests optimization of the site for a 5-carbon α-oxo dicarboxylic acid. Oxalate, α-Km, OAA, α-Kg, OG, α-Ka, and α-Kp, Figure 2, constitute a series of α-keto dicarboxylic acids and can be compared to α-Kg as such. The log of the affinity constant for each of the

keto acid analogues can be plotted against the chain length corresponding to numbers of C atoms, Fig. 6. The binding affinity of OAA and α-Ka, which are a methylene group shorter and longer than α-Kg, respectively, have decreased about 50-fold in affinity compared to α-Kg. Similarly, the binding affinity of α-Km and α-Kp, with two methylene groups shorter and longer than α-Kg, respectively have decreased about 200-fold in affinity, while in the case of oxalate, with three fewer methylene groups, the binding affinity has been decreased by about 360-fold. Thus, binding of a 3-carbon chain is optimal, and the distance between the C1-C2 unit and the C5 carboxylate is an important factor for determining the affinity of SDH for the α-keto acid substrate. However, of the 5 α-keto acid analogues of α-Kg, only α-Km and α-Ka can serve as an alternative substrate. The keto acid substrate binding pocket can thus accommodate the geometry of α-Ka, suggesting that the flexibility of the α-Ka side chain allows binding of the δ-carboxylate to the group on enzyme that interacts with the γ-carboxylate of α-Kg, while in the case of α-Km, the α-carboxylate is far enough away to eliminate its interaction with the same group. In addition, the α-oxo groups of α-Ka and α-Km are oriented such that hydride transfer from the nicotinamide ring of NADH is allowed.

Malonate, succinate, glutarate, and adipate, dicarboxylic acids, Figure 2, can also be considered as a series. Glutarate has the lowest K_i value, suggesting that a carboxylate at the 1 and 5 positions provides the tightest binding, consistent with the above consideration of α-keto acid analogues. Increasing or decreasing the chain length by a methylene or more decreases affinity by more than an order of magnitude. In addition, the inhibition constants of α-Kg and α-Ka, which have the α-oxo group, are decreased compared to those of glutarate and adipate, which have the same chain length, but no α-oxo group; the α-oxo contributes a factor of 10 in affinity. With malonate/α-Km as the inhibitors, there is no effect of the α-oxo group suggesting that the two carboxylates are likely bound to the α-carboxylate and α-oxo binding sites of α-Kg. Similarly, with succinate/OAA, an increase by a factor of 3 is observed for the α-oxo acid. Since the α-oxo contributes a factor of 10 in binding energy, the β-carboxylate of OAA must decrease the affinity by about 3-fold. This is likely a result either of binding the β-carboxylate to the α-oxo site or the hydrophobic nature of the site in the vicinity of C4 of α-Kg.

The importance of the C5 carboxylate group can be further illustrated by a series of α-keto analogues with hydrophobic side chains; the K_i or K_m values of α-Kiv, α-Kv, and α-Kb, Figure 2, are 36, 94, and 153 mM, respectively. However, all are alternative substrates, likely as a result of a lack of interaction with the group that binds the γ-carboxylate of α-Kg.

Finally, pyridine carboxylates provide information on the geometry of the bound α-Kg. If one assumes that the nitrogen atom of the pyridine ring mimics the oxygen atom of the α-keto group, a carboxylate in the 2-position is required. Addition of a second carboxylate, mimicking the γ-carboxylate of α-Kg can best be accommodated at the 4-position as in pyridine 2,4-dicarboxylate, which has the lowest K_i value (1.1 mM at pH 7.0). In addition, the K_i values of pyridine 2,4-dicarboxylate and glutarate are identical, within error, suggesting that the bulky aromatic ring does not affect binding when properly positioned. The distance between the two carboxylates of pyridine 2,4-dicarboxylate is 3.77 Å, very close to that of glutarate in its extended conformation, 3.83 Å (Cambridge Structural Database). Pyridine 2,3-dicarboxylate has a K_i value similar to those of malonate and succinate, which is not unreasonable, because the aromatic ring of the pyridine may orient these two carboxylates such that this part of structure mimics the 3- and 4-carbon dicarboxylic acids. Data suggest that the keto acid binding pocket is relatively large and flexible, and can accommodate the bulky aromatic ring of the pyridine.

Amino Acid Analogues

Inhibitory Keto Acid Analogues—SDH exhibits an absolute specificity toward its amino acid substrate L-lysine. D-Lysine is not a substrate, and binds with 5-fold lower affinity

compared with L-lysine, consistent with the stereospecificity. To gain insight into the structural requirements for amino acid binding, lysine analogues were tested for their effectiveness to inhibit the reaction. The frame of reference is the K_d for lysine from the central complex. The primary deuterium kinetic isotope effects on V and (V/K_{Lys}) at pH 7.0 are identical, within error, and thus the K_d value for L-lysine is identical to its K_m, i.e., 1.1 ± 0.2 mM (13). It is interesting to note that when pyruvate, a poor substrate, is used the K_m for lysine increases by about 50-fold, almost certainly reflecting a decreased affinity for lysine to the E-NADH-pyruvate complex. This is likely a result if the inability of pyruvate to generate the proper conformation. Unlike α-Kg, pyruvate cannot make the interaction of the γ-carboxylate.

Interestingly, when ornithine was substituted for lysine, the inhibition constant is increased by about 5-fold compared to the K_d of lysine, suggesting that the length of hydrophobic portion of amino acid carbon side chain is essential for optimal binding. L-Arginine and L-glutamine are poor inhibitors vs L-lysine, and L-asparagine causes no inhibition at 50 mM, indicating that the amino acid substrate binding site is hydrophobic, in agreement with previous results (4).

The inhibition constants for aliphatic amino acids, L-valine, L-isoleucine, L-methionine, L-norvaline, and L-leucine, as competitive inhibitors against L-lysine at pH 7.0, are increased by at least 2-fold compared to the value at pH 6.0, consistent with the previously reported pH dependence of the pK_i for leucine, which binds optimally at pH values below 6.0 (4). The pH dependence reflects a close proximity of the leucine hydrophobic side chain to an enzyme carboxylate. Hydrophobic amino acids with a linear 3 or 4 carbon side chain are good inhibitors.

L-Leucine binds to the amino acid binding site with a higher affinity than the natural substrate L-lysine, suggesting the binding pocket can accommodate a branch at the γ-carbon. Consistent with this idea, L-methionine, with a large S atom substituent at δ-carbon, has the same binding affinity as L-lysine. However, a branch at the β-carbon, as is present in L-valine and L-isoleucine, increases the inhibition constant dramatically, indicating a tighter binding site at the C1 and C2 positions.

Double Inhibition Studies

In the direction of saccharopine formation, the double-inhibition pattern obtained with OG and ornithine is shown in Fig. 5. The inhibition by ornithine alone is observed as the line at zero OG, reflecting the E-NADH-ornithine complex, while the inhibition by OG alone (ordinate) reflects the E-NADH-OG complex. The slope effect indicates the enhancement of inhibition as a result of synergism of binding between OG and ornithine. A value of 0.7 obtained for α suggests a 1.4-fold increase in the affinity of OG in the presence of ornithine, compared to its absence. Since OG and ornithine are competitive inhibitors of α-Kg and lysine, respectively, the observed synergism of binding between OG and ornithine suggests a slight synergism of binding between α-Kg and lysine. However, when its concentration was increased, OG began to compete with ornithine for the lysine binding site (data not shown), consistent with the previously proposed kinetic mechanism (3). In contrast, results obtained with pyridine 2,4-dicarboxylic acid and ornithine double inhibition gives a value of 2.4 for α, suggesting antagonism of binding. The difference in the double-inhibition patterns of OG and pyridine 2,4-dicarboxylic acid is due to difference in structure of the bulky pyridine and the optimally bound OG. The bulky aromatic ring of pyridine 2,4-dicarboxylic acid likely interferes with the binding of ornithine, while in the case of OG, structurally very similar to α-Kg, a better indication of the interaction of α-Kg and lysine is obtained.

Conclusion

Saccharopine dehydrogenase is the only enzyme in the α-aminoadipate pathway for lysine biosynthesis in fungi for which mechanistic data have been obtained and for which a structure has recently been determined (Albert M. Berghuis, personal communication). This allows an elucidation of the molecular basis for the specificity of SDH for all of its substrates. A survey of NADH, α-Kg, and lysine analogues has been undertaken to define the geometry of the active site and to identify functional groups on all three substrates important for substrate binding. The following can be concluded.

1. The majority of the binding energy of dinucleotides comes from the AMP portion of the molecule.
2. Distinctly different conformations are generated upon binding of the oxidized and reduced dinucleotides, and upon binding of NADH or NADPH.
3. The binding pocket for the nicotinamide ring is largely hydrophilic.
4. A side chain with 3 carbons (from the α-keto group up to and including the side chain carboxylate), with 2 carbons between the C1-C2 unit and the C5 carboxylate optimal for binding of α-keto acids; the α-oxo group contributes a factor of 10 in affinity.
5. The keto acid binding pocket is relatively large and flexible, and can accommodate the bulky aromatic ring of pyridine dicarboxylic acid and a negative charge at the C3 but not the C4 position.
6. The amino acid substrate binding pocket is hydrophobic, and the optimal length of the hydrophobic portion of the amino acid side chain is 3 or 4 carbons.
7. The amino acid binding pocket can accommodate a branch at the γ-carbon, but not at the β-carbon.

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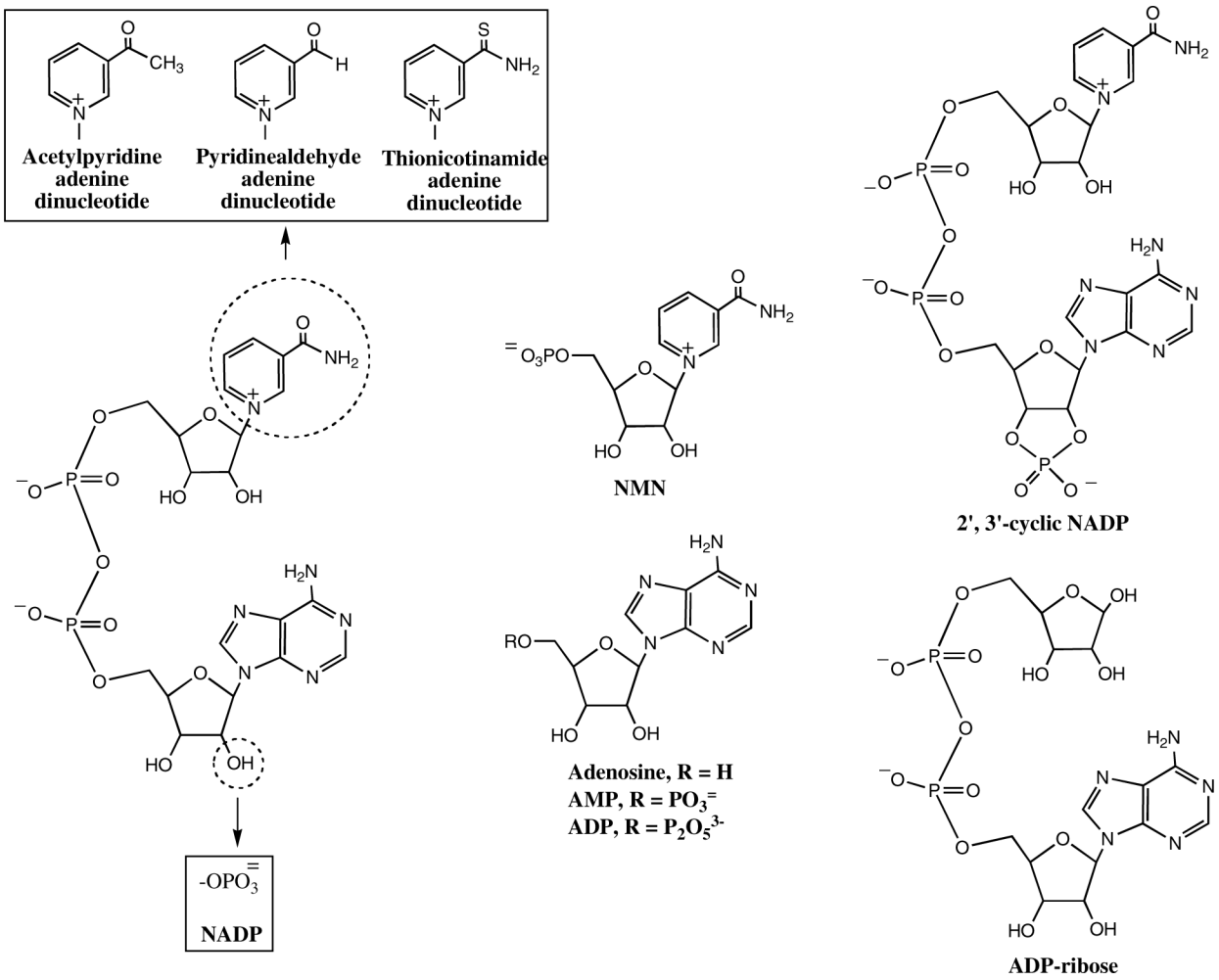


Figure 1.

Substrate and Inhibitory Analogs of NAD. Structures given in boxes exhibit substrate activity when they are used in place of NAD, while the other analogs are inhibitory.

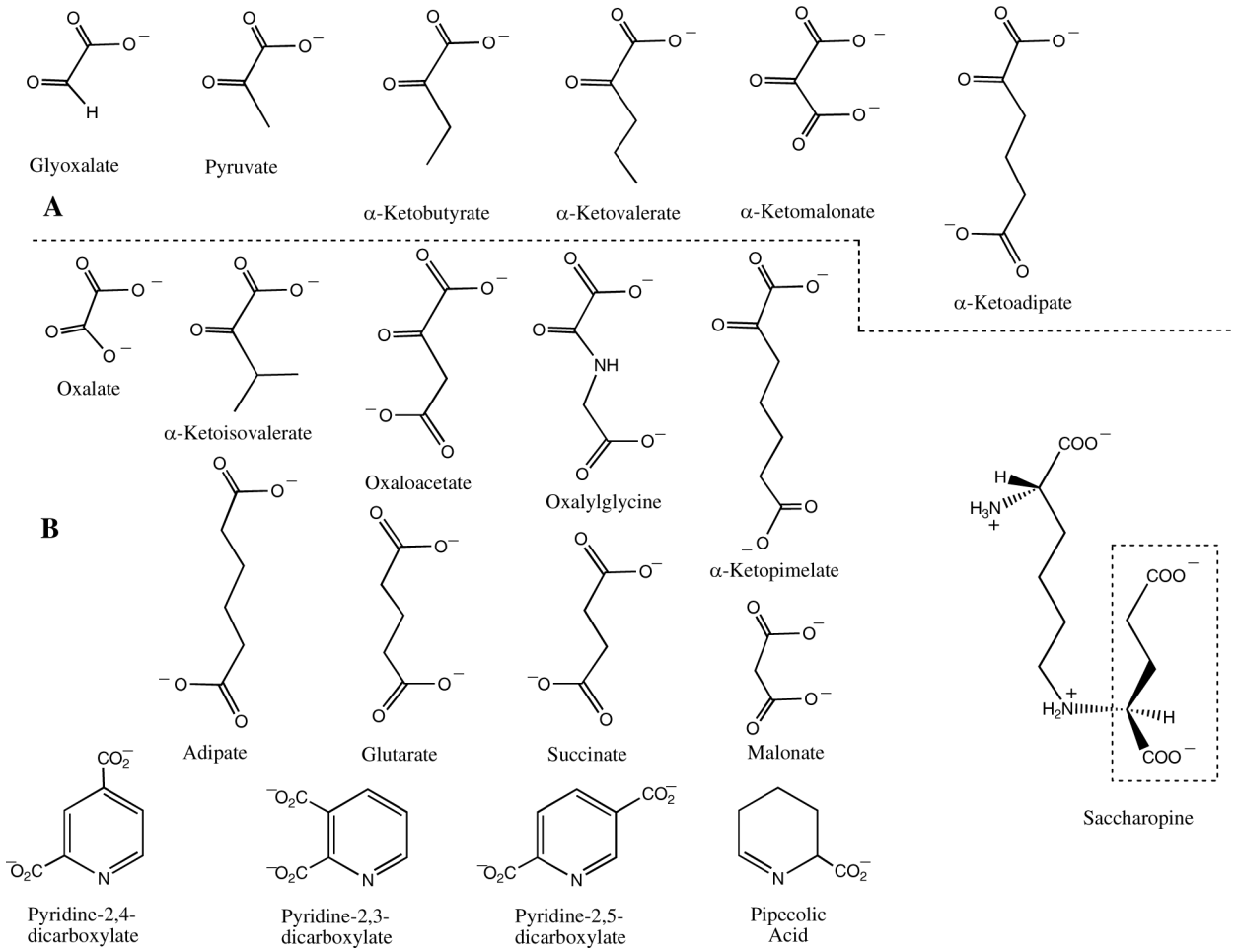


Figure 2.

Substrate and Inhibitory Analogs of α-Ketoglutarate or the glutamyl portion of saccharopine. A. Substrate analogs. B. Competitive inhibitors. The portion of saccharopine in the dotted box is mimicked by the inhibitors.

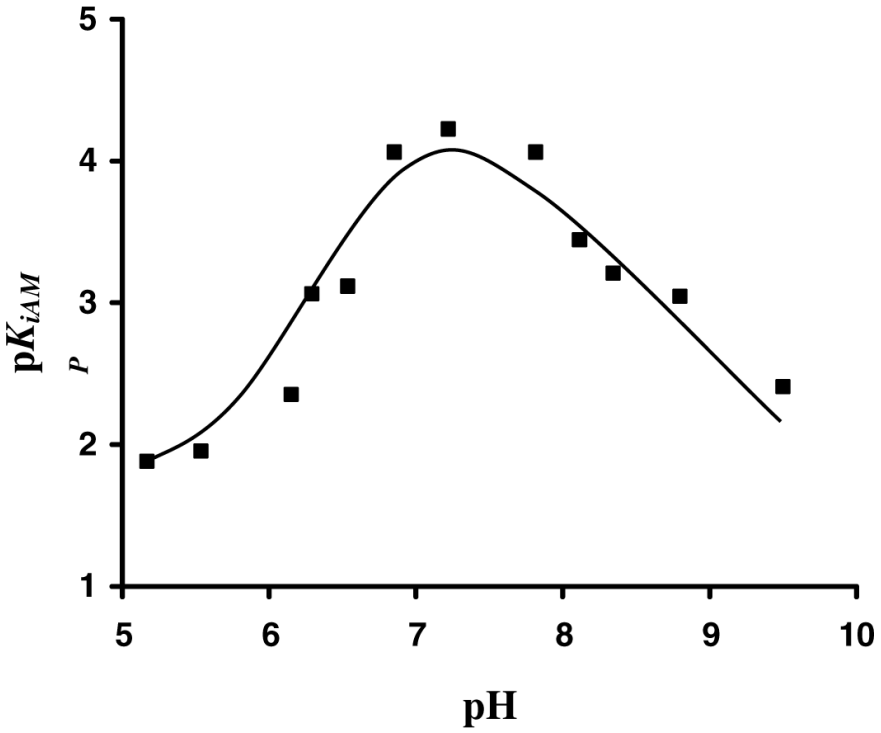


Figure 3.

pH dependence of the reciprocal of the inhibition constant for AMP. The curve is drawn by eye with pK_a values that fit the experimental data (points shown) calculated manually using eq 8. An average pK_a of about 7.1 is estimated for the groups observed; one group on enzyme must be protonated and two groups must be unprotonated, one from AMP and one from enzyme. Of the two that must be unprotonated, one of these gives a partial change so that the value of pK_i becomes constant at low pH.

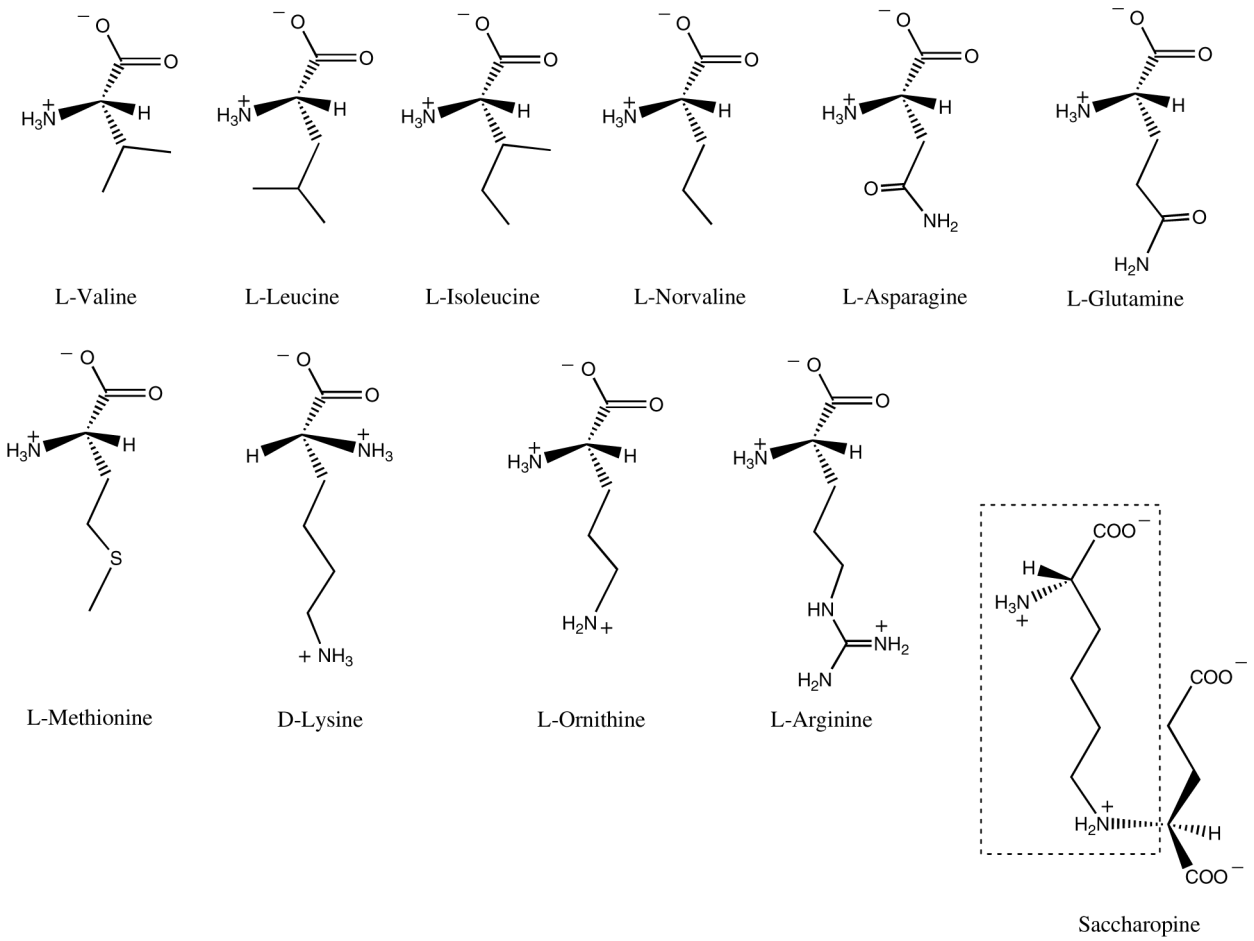


Figure 4.
Inhibitory Analogs of L-Lysine or the lysyl portion of saccharopine. The portion of saccharopine in the dotted box is mimicked by the inhibitors.

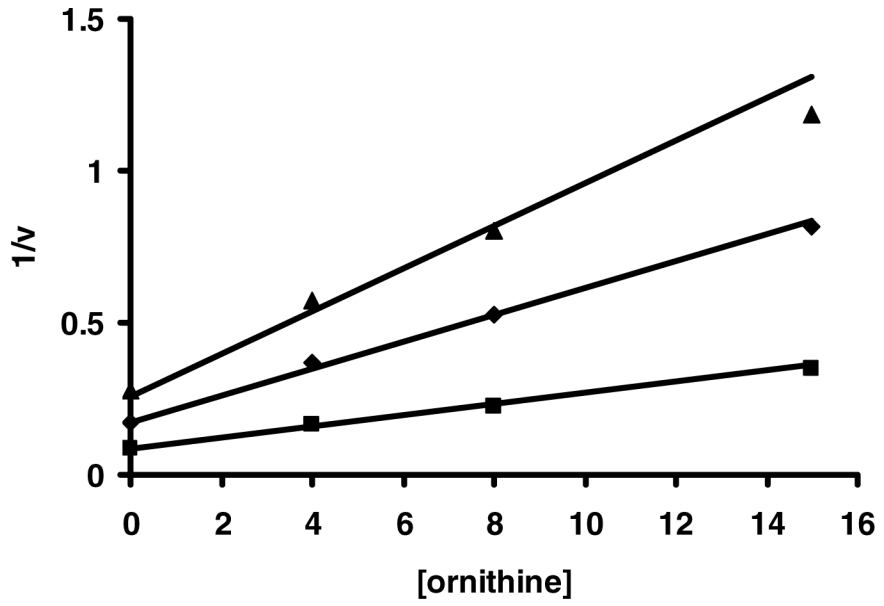


Figure 5.

Double inhibition by oxalylglycine and ornithine. Plot of the reciprocal initial rate versus the ornithine concentration (0−15 mM) at different levels of oxalylglycine [0 mM (ν), 0.05 mM (υ), and 0.1 mM (σ)]. The points are experimental, while the solid lines are theoretical based on a fit to eq 6. Values of v_0, the appK_i values for E-NADH-OG and E:NADH:ornithine complexes and α are 11.8 ± 0.2 μM/min, 0.050 ± 0.002 mM, 4.6 ± 0.2 mM, and 0.7 ± 0.1, respectively.

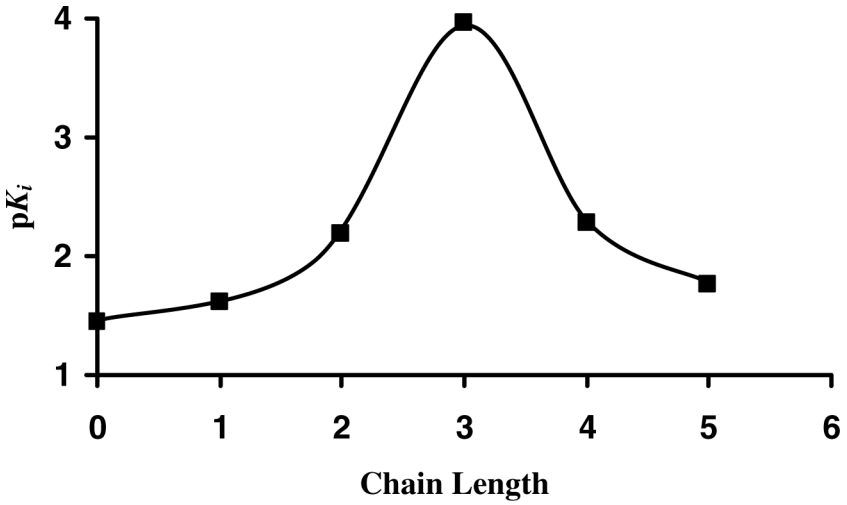
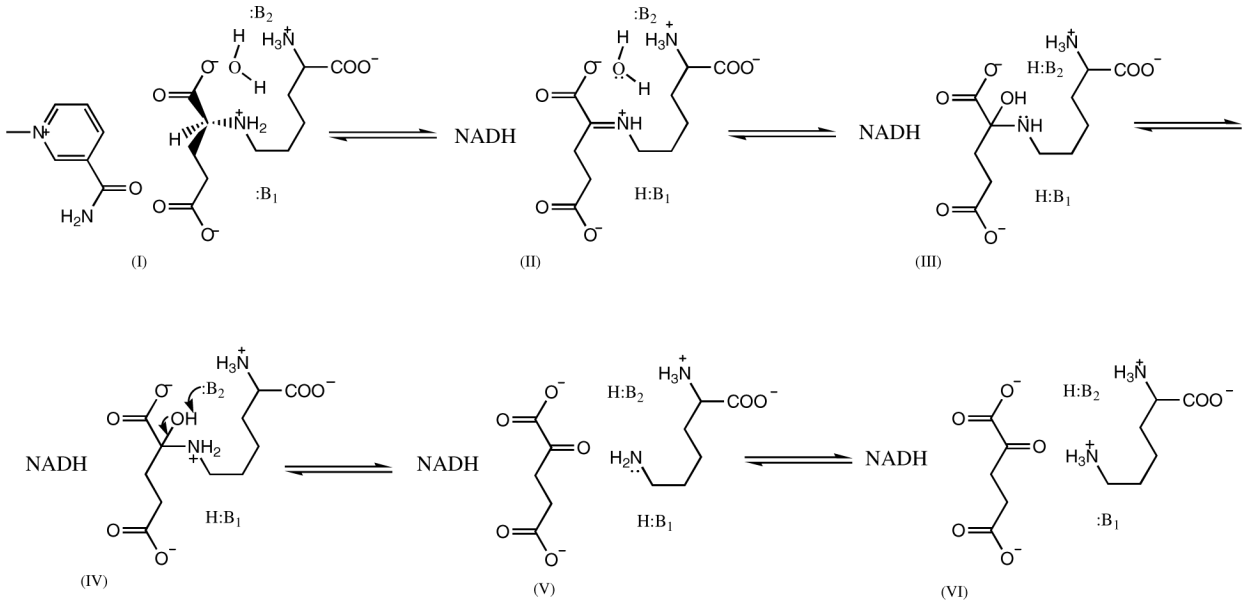


Figure 6.
Binding affinity of keto acid substrates against chain length from C3 and including the side chain carboxylate. From left to right are oxalate, α-Km, OAA, α-Kg, α-Ka, and α-Kp, respectively. Points are experimental values, and curve is drawn by eye.



Scheme 1.
Proposed Chemical Mechanism for Saccharopine Dehydrogenase.

Table 1

Kinetic Parameters for Alternative Coenzyme Substrates of SDH in the Direction of Saccharopine Formation.

	K_{ia} (mM)	V_{app} (μM/min)
NAD	1.1 ± 0.3 (K_{ia}) [a] 0.9 ± 0.1 (K_m) [a]	ND[d]
NADP	2.3 ± 0.4 [b] 5.7 ± 0.9 [c]	2.5 ± 0.2 [b]
3-APAD	8.9 ± 1.7 [b] 8 ± 1 [c]	63 ± 7 [b]
3-PAAD	0.88 ± 0.06 [b] 2.1 ± 0.4 [c]	10.4 ± 0.2 [b]
thio-NAD	0.48 ± 0.05 [b] 0.6 ± 0.1 [c]	11.5 ± 0.6 [b]

[a] value from reference (3) at pH 7.0; the values of K_{ia} and K_m are from a systematic analysis where the initial rate was measured as a function of both substrates.

[b] Data obtained at pH 7.55 and

[c] data obtained at pH 9.2. All K_{ia} are essentially the dissociation constant for the E-dinucleotide complex.

[d] Not determined.

Table 2

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

	NADH[a]	NADPH
$K_{coenzyme}$ (mM)	0.019 ± 0.002	0.48 ± 0.17
$K_{\alpha\text{-}Kg}$ (mM)	0.11 ± 0.03	12 ± 4
K_{Lys} (mM)	1.1 ± 0.2	22 ± 9
$K_{icoenzyme}$ (mM)	0.018 ± 0.004	0.04 ± 0.01
$K_{i\alpha\text{-}Kg}$ (mM)	0.6 ± 0.1	6.0 ± 1.4
K_{iLys} (mM)	NA[c]	9.4 ± 2.0
V/E_t (s^{-1})	20 ± 1	22 ± 6
$V/K_{coenzyme}\ E_t$ ($M^{-1}{\cdot}s^{-1}$)	$(1.6 \pm 0.2) \times 10^6$	$(4.6 \pm 2.0) \times 10^4$
$V/K_{\alpha\text{-}Kg}\ E_t$ ($M^{-1}{\cdot}s^{-1}$)	$(2.8 \pm 0.7) \times 10^5$	$(1.8 \pm 0.8) \times 10^3$
$V/K_{Lys}\ E_t$ ($M^{-1}{\cdot}s^{-1}$)	$(2.5 \pm 0.4) \times 10^4$	$(1.0 \pm 0.5) \times 10^3$

Product and Deadend Inhibitor	Varied Substrate	K_i (mM) pH 7.0	Inhibition Pattern
NAD	NADH	1.56 ± 0.03[a]	C
NADP	NADPH	3.5 ± 0.6	C
L-leucine	NADPH	5.6 ± 0.3	UC
L-leucine	NADH[b]	69 ± 2	UC

[a] value from reference at pH 7.0 (3)

[b] For pyruvate reaction.

[c] Not applicable.

Table 3

Kinetic Parameters of SDH in the Direction of Saccharopine Formation using Keto Acid Substrates at pH 7.0.

Substrate	K_m (mM)	V/E_t (s^{-1})	V/KE_t (M^{-1} s^{-1})
α-Ketoglutarate[a]	0.11 ± 0.03	20 ± 1	(2.8 ± 0.7) × 10^5
Pyruvate[b]	4.1 ± 0.2	2.55 ± 0.02	381 ± 3
Glyoxylate[c]	6.4 ± 0.6	1.85 ± 0.09	289 ± 31
α-Ketobutyrate[c]	153 ± 34	27.4 ± 4.8	179 ± 31
α-Ketovalerate[c]	94 ± 13	9.5 ± 0.9	101 ± 10
α-Ketomalonate[c]	24 ± 1	12.5 ± 0.5	521 ± 21
α-Ketoadipate[c]	5.3 ± 0.2	4.43 ± 0.05	836 ± 9

[a] value from reference (3)

[b] K_m value of lysine for pyruvate reaction is 62 ± 1 mM

[c] The lysine concentrations used are 225 mM and 40 mM for glyoxylate and α-Kv reactions, respectively, and 20 mM for α-Kb, α-Km, and α-Ka reactions.

Table 4

Isotope Effects for SDH at pH 7.0.

Isotope Effects	α-Kg[a]	Pyruvate
^{D}V	1.45 ± 0.07	1.29 ± 0.09
$^{D}(V/K_{NADH})$	0.92 ± 0.08	2.2 ± 0.1
$^{D}(V/K_{keto\ acid})$	1.9 ± 0.1	1.9 ± 0.1
$^{D}(V/K_{Lys})$	1.56 ± 0.05	2.3 ± 0.2

[a] value from reference (3).

Table 5

Inhibition Constants of NAD analogue inhibitors at pH 6.9 in the Direction of Saccharopine Formation.[a]

Substrate/Inhibitor	K_i (mM)
NADH	0.018 ± 0.004[b]
Adenosine	0.42 ± 0.04
AMP	0.055 ± 0.006
ADP	0.093 ± 0.006
ADP ribose	0.29 ± 0.01
NMN	7.2 ± 0.6
NADP	1.2 ± 0.4
2′,3′-cyclic NADP	1.5 ± 0.1
NADP[c]	3.5 ± 0.6
NAD	1.56 ± 0.03[a]

[a] Data were obtained at pH 6.9, the pH where AMP has its maximum affinity.

[b] Value from reference (3); the varied substrate is NADH with the exception of

[c] where NADPH is the varied substrate.

Table 6

Inhibition Constants of α-Kg Substrate Analogue Inhibitors at pH 7.0 in the Direction of Saccharopine Formation.

α-Kg analogue inhibitors	**K_i (mM)**
Oxalate	**36 ± 4**
α-Keto Dicarboxylic Acids	
α-Ketomalonate[a]	24 ± 1
Oxaloacetate,[bd]	6.4 ± 0.5
α-Ketoglutarate[c]	0.6 ± 0.1
Oxalylglycine[c]	0.100 ± 0.002
α-Ketoadipate[a]	5.3 ± 0.2
α-Ketopimelate	18 ± 2
Dicarboxylic Acids	
Malonate	24 ± 2
Succinate	21 ± 1
Glutarate,[bd]	1.0 ± 0.1
Adipate	50 ± 2
Other	
α-Ketoisovalerate	36 ± 1
Pyridine 2,4-dicarboxylate	1.10 ± 0.01
Pyridine 2,3-dicarboxylate	18.3 ± 0.4

[a] Values are K_m for α-Km and α-Ka, and are likely equal to the dissociation constants for the E-NADH-lysine-keto acid complex given the decrease in V/E_t and increase in K_m compared to data with α-Kg.

[b] Value from reference (3)

[c] The value for α-Kg is for the E-NADH-α-Kg complex; the K_m for α-Kg is 0.11 ± 0.03 mM (3).

[d] OAA and glutarate are NC inhibitors (3). The slope inhibition constant, which reflects competitive inhibition vs α-Kg is reported.

Table 7

Inhibition Constants of Lysine Substrate Analogue Inhibitors in the Direction of Saccharopine Formation.

dead-end inhibitor	K_i (mM) pH 6.0	K_i (mM) pH 7.0
L-Lysine	1.7 ± 0.2 [a]	1.1 ± 0.2 [a]
D-Lysine	ND[c]	5.5 ± 0.4
L-Ornithine	3.6 ± 0.1	5.0 ± 0.1
L-Valine	62 ± 5	140
L-Norvaline	0.71 ± 0.04	1.13 ± 0.05
L-Methionine	0.96 ± 0.03	3.12 ± 0.08
L-Leucine	0.125 ± 0.002 [b]	0.44 ± 0.03 [b]
L-Isoleucine	10.7 ± 0.4	22 ± 2.0
L-Arginine	ND	16.4 ± 0.4
L-Glutamine	ND	16.0 ± 0.8
L-Asparagine	ND	No inhibition

[a] K_m values for lysine from reference (3); values are equal to K_d values for central complex (see discussion of isotope effects).

[b] Values from reference (3).

[c] Not determined