Reversible Reduction of an α-Imino Acid to an α-Amino Acid Catalyzed by Glutamate Dehydrogenase: Effect of Ionizable Functional Groups†

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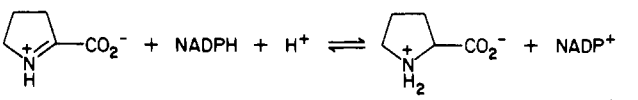
ABSTRACT: The glutamate dehydrogenase catalyzed reduction of Δ^1-pyrroline-2-carboxylic acid (PCA; an α-imino acid) with reduced nicotinamide adenine dinucleotide phosphate (NADPH) to give L-proline and $NADP^+$ is employed as a model for the redox step of the corresponding enzyme-catalyzed reductive amination of α-ketoglutarate. We demonstrate the reversibility of the model reaction and measure its equilibrium constant. The pH profiles for the model reactions show that the active substrates are the N-protonated imino acid in one direction and the proline anion with a neutral amino group in the other. The V/K value for the imino acid reduction is enhanced by a group Z of $pK = 8.6$ in the enzyme–NADPH complex, while that for the proline reaction is unaffected by any such group in the enzyme–$NADP^+$ complex. The following conclusions emerge from a comparison of the pH dependence of the rates for the model reactions with that for the oxidative deamination of L-glutamate [Rife, J. E., & Cleland, W. W. (1980) *Biochemistry 19*, 2328]. (1) The N-protonated form of α-iminoglutarate and the conjugate base of glutamate are the active substrates. (2) The redox step is not sensitive to the protonation state of the groups that catalyze the hydrolysis of bound α-iminoglutarate. (3) The group Z, which facilitates the PCA reaction, plays no role in the binding of α-ketoglutarate. We propose a chemical mechanism for the glutamate reaction where an unprotonated enzyme group of $pK = 5.2$ in enzyme–NADPH catalyzes the conversion of the α-iminoglutarate to the carbinolamine. A protonated enzyme group of $pK = 8$ in enzyme–$NADP^+$ is required for the conversion of the carbinolamine to bound α-ketoglutarate.

Glutamate dehydrogenase catalyzes the reversible oxidative deamination of L-glutamate. Several lines of evidence support the occurrence of an α-iminoglutarate as a reactive intermediate in the reaction (Brown et al., 1978; Fisher et al., 1982; Fisher & Viswanathan, 1984). However, kinetic studies, which include steady-state and pre-steady-state rate measurements, have proved to be of limited value in yielding mechanistic information about the individual chemical steps because of the kinetic complexity of the reaction. Indeed, the slow step in the steady-state oxidative deamination of glutamate is the release of products from enzyme ternary complexes (di Franco, 1974; Silverstein & Sulebele, 1973; Colen et al., 1975) while that in the opposite direction is not well characterized. The burst of reduced nicotinamide adenine dinucleotide phosphate (NADPH) disappearance observed by Jallon et al. (1975) for the reductive amination of α-ketoglutarate was interpreted to include both the imine formation and NADPH oxidation steps. Similarly, for the oxidative deamination of glutamate the burst phase (Iwatsubo & Pantaloni, 1967), which appears to include the hydride transfer event, may well contain contributions from the subsequent imine hydrolysis steps (Fisher et al., 1970; Colen et al., 1972, 1977).

An attractive approach by which the redox step could be studied with minimal kinetic complexity is provided by a model reaction—the glutamate dehydrogenase catalyzed reduction of Δ^1-pyrroline-2-carboxylic acid (PCA) to proline (Fisher et al., 1982). This model reaction corresponds to only the redox step of the ketoglutarate reaction in that both reactions involve the reduction of an α-imino acid to an α-amino acid. We have previously reported that the model reaction takes place at the same active site as the ketoglutarate reaction with the enzyme–NADPH complex being several hundred times more effective than NADPH in reducing PCA (Fisher et al., 1982).

We now show that the enzymatic reduction of PCA, eq 1, is reversible and report the pH dependence of rates in the forward and reverse directions. From a comparison of the



(1)

PCA and proline profiles with those for norvaline and glutamate (Rife & Cleland, 1980) we are able to sort out the enzyme groups that affect the hydrolysis of enzyme-bound α-iminoglutarate as distinct from those that are involved in its reduction to glutamate.

Experimental Procedures

Materials. L-Proline and D-proline from Sigma and DL-proline from Aldrich were purified by repeated crystallization from aqueous ethanol. DL-Proline-*2-d* purchased from Merck Sharp and Dohme was crystallized from ethanol and ethanol–ether. α-Keto-δ-aminovaleric acid was synthesized as the hydrochloride salt by the method of Hasse & Wieland (1960). This compound when dissolved in water at pH >4 spontaneously cyclizes to PCA (Cabello et al., 1964; Macholan & Vencalkova, 1963). NADPH from Boehringer-Mannheim and $NADP^+$ from Sigma were used without further purification. Bovine liver L-glutamate dehydrogenase was obtained as an ammonium sulfate suspension from Boehringer-Mannheim and treated with Norit A as described elsewhere (Cross & Fisher,

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1970). The enzyme solution (0.1 M phosphate buffer, pH 7.60) was concentrated by ultrafiltration under nitrogen. The concentrations of the stock enzyme solution, NADPH, and $NADP^+$ were determined spectrophotometrically (Johnson et al., 1981). NADH-*4,4-d_2* was prepared according to published procedures (Brown & Fisher, 1976).

Kinetic Procedure. Kinetic runs were followed in a Hewlett-Packard 8450A UV/vis spectrophotometer as described previously (Srinivasan et al., 1982). The solutions in the cuvettes were kept stirred at all times, and their temperature was maintained at 25.0 ± 0.1 °C with a Hewlett-Packard 89100A temperature controller. The reactions were initiated by adding to 2 mL of the thermostated solution 20–100 μL of the enzyme stock solution and 10 μL of the coenzyme solution. The pH of the solutions measured before and after rate measurements remained constant.

Rates of the enzymatic reduction of PCA were measured by following the disappearance of NADPH, while those of the enzymatic oxidation of L-proline were measured by following the production of NADPH, both at 340 nm. The rates were computed from the initial part of the reaction ($\Delta A \sim 0.01$) where a plot of absorbance vs. time was linear. The observed rates for the enzymatic reduction of PCA were corrected for the nonenzymatic reaction occurring concurrently by measuring the rate of disappearance of NADPH under identical conditions in the absence of the enzyme. The blank reaction, which includes contributions from both the nonenzymatic reduction of PCA (Srinivasan et al., 1982; Meister et al., 1957) and general-acid-catalyzed decomposition of NADPH (Norris & Stewart, 1977), is usually less than 10% of the observed rates. In the reverse direction (eq 1), however, no reaction was observed in the absence of the enzyme. For kinetic studies at pH <6 and at pH >9 the enzyme from the reaction mixture was assayed for activity before and after rate measurements. Only those pH regions where the enzyme activities remained constant are included here.

Reaction Conditions. All measurements were made at 25.0 ± 0.1 °C in 0.1 M tris(hydroxymethyl)aminomethane (Tris) and 0.01 M phosphate buffer. Whenever it was necessary to change the concentration of L-proline, the total proline concentration was kept constant by the addition of D-proline. The solutions were adjusted to the desired pH values with concentrated hydrochloric acid or potassium hydroxide.

Results

Reversibility of the PCA Reduction Reaction. When glutamate dehydrogenase was added to a solution of L-proline and $NADP^+$ (pH 7.5–10.0), a time-dependent increase in the absorbance at 340 nm was observed. There was a rapid depletion of this absorbance on the addition of α-ketoglutarate and ammonia, demonstrating that NADPH is produced as one of the products of the reaction.[1] A kinetic deuterium isotope effect of <3 (25 °C, pH 9.45) was obtained from rate measurements with DL-proline and DL-proline-*2-d*. The observed reduction of $NADP^+$ to NADPH and the occurrence of a primary deuterium isotope effect show that there is a net hydride transfer from the amino acid in the rate-determining step. Consequently, the imino acid, PCA, must be the intermediate product of the reaction.

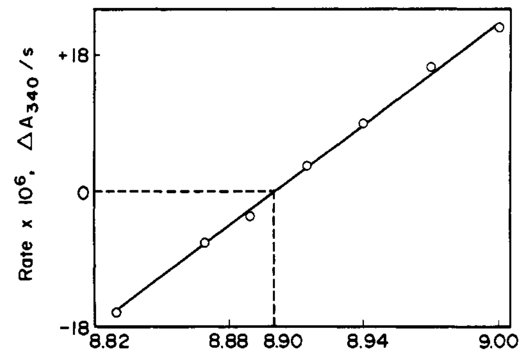
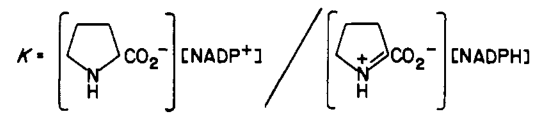


FIGURE 1: Rate of appearance or disappearance of NADPH as a function of pH. [E] = 0.4 mg/mL, [L-proline] = 1.0 M, [PCA] = 72 mM, [NADPH] = 150 μM, [$NADP^+$] = 253 μM, [Tris] = 0.1 M, and [phosphate] = 0.01 M. The pH, 8.90, at which the system is at equilibrium is determined by interpolation (dotted line).

The equilibrium constant K (as determined in eq 2) for PCA–proline interconversion was obtained by observing the rate of NADPH production or depletion at various pH values at fixed amounts of PCA, L-proline, glutamate dehydrogenase, $NADP^+$, and NADPH. Since PCA, which has a pK_{SH^+} value



(2)

of 6.05 (Srinivasan et al., 1982), and the conjugate base of proline are present as minor species over the pH region 8.83–9.00, varying the pH changes the concentrations of both of these species simultaneously. Consequently, as the pH of the solution is lowered, the rate of NADPH appearance gradually diminishes to a point where the reaction reverses its direction, causing NADPH to disappear. The value of pH_{eq}, that pH value at which the absorbance at 340 nm is invariant with time, was determined by interpolation as shown in Figure 1. Under the experimental conditions the reactants and products are at equilibrium at pH_{eq} 8.90 ± 0.02, from which K was calculated. We have determined the equilibrium conditions over the pH_{eq} range 7.9–9.2 at 1 M proline concentration. We found that the K values which involve the concentrations of the protonated imine and unprotonated proline are indeed independent of pH and the concentrations of PCA, NADPH, and $NADP^+$. An average value of 280 ± 50 was obtained for the equilibrium constant.

Dependence of Rate on the Concentration of Reactants. The rate of oxidation of L-proline by $NADP^+$ is proportional to the enzyme concentration in the pH region 7.5–10.0. The extrapolated rate at zero enzyme concentration is virtually zero. The rate is also linear with the concentration of proline (up to 1.3 M proline) over the pH region 9.0–10.0.

Dissociation Constants for Enzyme–Coenzyme Complexes. The value of K_{NADPH} at pH 7.10 was determined from the rates obtained at a constant PCA concentration but varying concentrations of NADPH to be 14 ± 3 μM. The K_{NADPH} value is in agreement with the dissociation constant for the enzyme–NADPH complex, $K_{i\,NADPH}$ of 20 ± 5 μM, obtained calorimetrically under the same experimental conditions except that PCA was replaced by an equimolar amount of potassium chloride. However, the K_{NADP^+} value calculated from the proline oxidation rates depends upon the range of coenzyme concentration employed (Engel & Dalziel, 1969), the value becoming progressively larger as the concentration range of $NADP^+$ is increased (K_{NADP^+} 2 μM to 1 mM at pH 8.9 over

[1] The production of NADPH is not due to any trace impurity of L-glutamate since (i) the rate is independent of the number of times L-proline was crystallized and (ii) DL-proline synthesized chemically has half the reactivity of the L isomer produced from biological sources; D-proline, which under the same conditions is unreactive (<0.1% the reactivity of the L isomer), is neither a substrate nor an inhibitor of the glutamate dehydrogenase reaction.

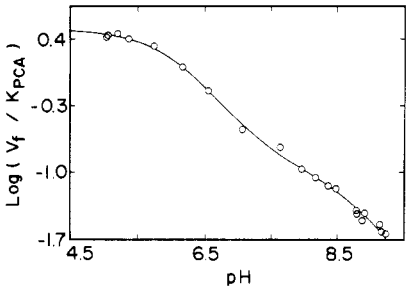


FIGURE 2: pH dependence of V_f/K_{PCA}. V/K is expressed in M^{-1} s^{-1}. The assays contained 53.6 mM PCA, 0.1 M Tris, and 0.01 M phosphate. [Enzyme] was varied from 0.14 to 0.41 mg/mL and [NADPH] from 150 to 300 μM. The curve is a fit to eq 4 with pK_1 = 5.96 ± 0.06, pK_2 = 8.63 ± 0.08, A = 3.1 ± 0.2 M^{-1} s^{-1}, and B = 47 ± 7 M^{-1} s^{-1}.

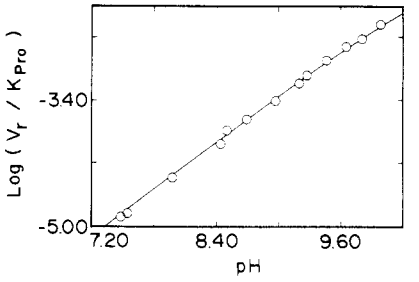


FIGURE 3: pH dependence of V_r/K_{Pro}. V/K is expressed in M^{-1} s^{-1}. The assays contained 1.0 M L-proline and 0.01 M phosphate. [Enzyme] was varied from 3 to 1.0 mg/mL and [$NADP^+$] from 1 to 2 mM. The curve was fit to eq 6 with pK_3 = 10.64 and D ~0.02 M^{-1} s^{-1}.

the [$NADP^+$] ranges 5–80 μM and 40 μM–3.4 mM, respectively).

pH Dependence of PCA Reduction Rates. The PCA reduction rates v_f^{obsd} at various pH values were calculated from the observed rates at [NADPH] of 150–300 μM extrapolated to infinite concentration of NADPH. They were then converted to V_f/K_{PCA} values[2] by eq 3, where $[PCA]_t$ and $[ERH]_t$

$$v_f^{obsd} = (V_f/K_{PCA})[PCA]_t[ERH]_t \quad (3)$$

represent respectively the analytical molar concentration of PCA and the micromolar concentration of the glutamate dehydrogenase–NADPH complex. A plot of log (V_f/K_{PCA}) against pH fitted by eq 4 (where A and B are constants) is shown in Figure 2.

$$\frac{V_f}{K_{PCA}} = \frac{A}{(1 + K_1/[H^+])(1 + K_2/[H^+])} + \frac{B}{(1 + K_1/[H^+])(1 + [H^+]/K_2)} \quad (4)$$

pH Dependence of Proline Oxidation Rates. We have derived V_r/K_{Pro} ratios[2] according to eq 5, where $[Pro]_t$ and $[EOH]_t$ represent respectively the total molar concentration of proline and the micromolar concentration of the enzyme–$NADP^+$ complex. The values of v_r^{obsd} were obtained by

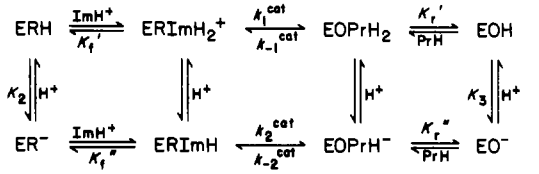
$$v_r^{obsd} = (V_r/K_{Pro})[Pro]_t[EOH]_t \quad (5)$$

extrapolating the observed rates at [$NADP^+$] of 1 and 2 mM to infinite [$NADP^+$]. A plot of log (V_r/K_{Pro}) against pH is shown in Figure 3, where the value of D is determined according to eq 6 assuming a pK_3 value of 10.64, the dissociation

$$V_r/K_{Pro} = D/(1 + [H^+]/K_3) \quad (6)$$

[2] The term V/K is used to denote the fact that under the experimental conditions the concentration of the carboxylic substrate is much less than its K_m value.

Scheme I



constant of proline. The results are consistent with the assignment that a group with a pK_3 of 10.64 must be unprotonated for the proline oxidation reaction to proceed.[3]

Discussion

The pH dependence of the enzyme-catalyzed reduction of PCA by NADPH and that of the oxidation of L-proline by $NADP^+$ are described by the general phenomenological equations (4) and (6). We now consider the mechanistic implications of the various parameters of those equations. In this we are aided by two kinetic constraints: (1) we observe an isotope effect of 3.9 ± 0.2 for the reduction of PCA with NADH-*4,4-d*$_2$ at pH 6.70, and (2) we observe an isotope effect of >3 for proline oxidation at pH 9.45 using DL-proline-*2-d* and $NADP^+$. Consequently, the redox step in each direction must be at least partly rate limiting.

Active Substrates in the Enzymatic Model Reaction. The minimal mechanism for the PCA–proline reaction is given in Scheme I, where the participation of an enzyme–coenzyme–substrate ternary complex in each direction is assumed. Here, EH, R, O, ImH^+, and PrH_2^+ represent glutamate dehydrogenase, NADPH, $NADP^+$, PCA, and L-proline, respectively. Each of the constants K_f', K_f'', K_r', and K_r'' represents the dissociation constant of the appropriate ternary complex. Considering the reaction first from the direction of PCA reduction, we note that the pH dependence of V_f/K_{PCA} shown in Figure 2 demonstrates that a group with a pK of 6.0 must be protonated. The inflection seen at pH 7.45 arises as a result of a change in the major rate-contributing step at pH >7.45 and probably involves an additional group of pK = 8.6 in the unprotonated form. Since the imino group of PCA has a pK of 6.05, we assign the group with a pK of 6 to PCA. Thus, for PCA to bind and react, the imino group must be protonated over the entire pH region studied (5.3–9.3). Consequently, the iminium ion is at least 2000 times more reactive than the imine itself in the enzymatic reaction, just as it is in the nonenzymatic reaction (Srinivasan et al., 1982).

The observed pK of 8.6 must be attributed to some functional group on the enzyme–NADPH complex, which we define as ZH^+. At pH <7.45 the major contributor to the V_f/K_{PCA} term is the protonated form of this group, ZH^+, while at higher pH values it is Z. Constants A and B in eq 4, corresponding to k_1^{cat}/K_f' and k_2^{cat}/K_f'', reflect the effect of ZH^+ and Z on the enzymatic reduction of the iminium ion. It is apparent that the iminium ion binds and reacts about 15 times more effectively when Z is unprotonated than when it is protonated. The pH dependence of proline oxidation rates shown in Figure 3 suggests that the conjugate base of proline is the active reductant. However, the protonation state of the group ZH^+, which affects the V_f/K_{PCA} values, has no detectable influence on V_r/K_{Pro} values. Since K in eq 2 is pH independent, the differing sensitivity of PCA and proline reactions to the ionic state of Z implies that the ratio $K_{i\,NADP^+}/K_{i\,NADPH}$ must be pH dependent.

[3] The same dependence of V_r/K_{Pro} values on pH was observed with rates obtained at [$NADP^+$] of 1 and 2 mM.

Why Are the PCA and L-*Proline Reactions Affected Differently by the State of Protonation of Group Z*? Two quite different answers to this question are both consistent with the experimental evidence now on hand: (1) The pK of group ZH^+, which has a value of 8.6 in the enzyme–NADPH complex, may be shifted in the enzyme–$NADP^+$ complex to some value that is either so very high or so very low that the state of protonation of this group remains unchanged throughout the experimentally accessible range of pH 7.5–10.0. In this case group Z would in fact affect both the PCA and proline reactions, but its effect on the proline reaction would show no pH dependence. (2) Alternatively, it is possible that group Z does not affect the L-proline reaction at all. We have already pointed out that there is a difference in charge between the reactant species in the two directions; the imino nitrogen atom of PCA is protonated and carries a positive charge, while the amino group of proline must be unprotonated and neutral. Consequently, one would expect charge repulsion between ZH^+ and the iminium ion in the enzyme–NADPH–PCA complex but not between Z or ZH^+ and the neutral amino group in the enzyme–$NADP^+$–proline anion complex. Protonation of Z then will produce a lower concentration of PCA ternary complex but will be expected to facilitate the hydride transfer step itself. The modest 15-fold decrease by ZH^+ over Z on the V_f/K_{PCA} term is thus ascribable to these opposing effects in the magnitudes of V_f and $1/K_{PCA}$ terms. The lack of any interaction by Z or ZH^+ in the proline anion ternary complex would lead to proline oxidation rates that are independent of the ionic state of Z.

Comparison of the Model Reaction to the α-Ketoglutarate Reaction. Thus far we have considered those mechanistic conclusions that can be drawn specifically from the studies on the glutamate dehydrogenase catalysis of the PCA–proline reactions. This enzyme of course also catalyzes the reversible oxidative deamination of mono- and dicarboxylic α-amino acids. It appears that these reactions involve imine intermediate complexes corresponding to the initial substrate complex in the PCA reaction.

The V/K profile for glutamate reflects the pK's in enzyme–$NADP^+$ of those ionizable groups that influence the binding of the amino acid to this binary complex, the oxidation of enzyme–$NADP^+$–glutamate to bound α-iminoglutarate, and the subsequent hydrolysis producing ammonia and the enzyme–NADPH–α-ketoglutarate complex. However, the V/K profiles for the model reactions reported here are free of those groups that are involved in the hydrolysis of the enzyme-bound α-imino acid. Hence, we will compare the V/K profiles for the glutamate reaction and the model reactions to identify the steps with which specific enzyme groups are associated.

Rife & Cleland (1980) have identified a number of ionizable groups in the glutamate dehydrogenase–coenzyme complexes that are responsible for the binding and reacting of dicarboxylic and monocarboxylic substrates and have inferred the presence of several others. The groups identified by them, their pK values, and the protonation states required for the reaction are listed in Table I. The following conclusions regarding the involvement of functional groups emerge from a comparison of our findings with those of Rife & Cleland (1980).

Group YH^+. The results cited in Table I indicate that this group must be protonated in order to permit the binding of the 5-carboxylate group of either α-ketoglutarate or L-glutamate. On the other hand, group Y must be unprotonated to permit the binding of the monocarboxylic substrates, α-ketovalerate or norvaline. These facts imply a geometrically close steric relationship between the location of the Y group on the enzyme–coenzyme complexes and the C-5 position of the bound acyclic substrates. Therefore, the observed insensitivity of V_f/K_{PCA} and of V_r/K_{Pro} values to the state of protonation of Y is most easily ascribed to the absence of any moiety of the bound cyclic substrates close to the group Y in the enzyme–coenzyme complex.

Table I: Enzyme Groups in Enzyme–NADPH and Enzyme–$NADP^+$ Complexes, Identified by Rife & Cleland (1980)

group[a]	pK in enzyme–NADPH for binding α-ketoglutarate	pK in enzyme–$NADP^+$ from $V/K_{glutamate}$ data
HA	5.2 (p)	5.2 (u)[c]
YH^+[b]	7.9 (p)	9.0 (p)
XH^+	>10.5 (p)[c]	8 (u)

[a] Rife and Cleland have assumed HA to be a carboxylic acid and XH^+ and YH^+ to be lysines. [b] This group must be protonated for binding dicarboxylates, α-ketoglutarate, and glutamate but must be unprotonated for binding monocarboxylates, α-ketovalerate, and norvaline. [c] Refers to inferred pK's; p = protonated; u = unprotonated.

Ionic States of Imino Acid and Amino Acid Substrates. The fact that PCA binds and reacts in the iminium ion form over the entire pH region studied (5.0–9.2) implies that the enzyme-bound α-iminoglutarate in the ketoglutarate reaction undergoes oxidation to glutamate in the N-protonated form.

The pH dependence of V_r/K_{Pro} values (Figure 3) suggests that the conjugate base of proline is the active substrate in the proline reaction. Consequently, we conclude that, in the oxidative deamination of L-glutamate, the amino acid binds and reacts as the conjugate base.[4] The observed pH dependence of $V/K_{glutamate}$ values would be accounted for by considering that X must be protonated [rather than unprotonated as Rife & Cleland (1980) have postulated] for glutamate to bind and react.[4]

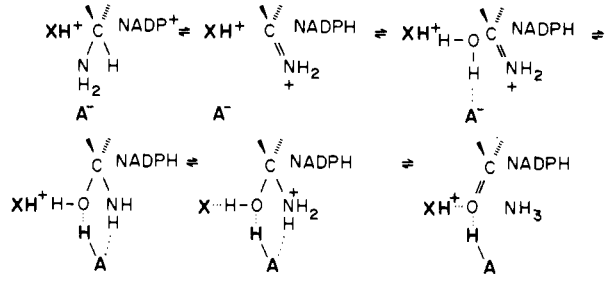
Group HA. The protonated form of this group is required for binding keto acids to enzyme–NADPH. However, the PCA profile clearly demonstrates that the enzymatic reduction of the imino acid is unaffected by the protonation state of HA. It thus appears that this group plays no role in the dehydrogenation step of the glutamate reaction.

Group XH^+. We have proposed that this group of pK = 8 in enzyme–$NADP^+$ must be protonated for the conjugate base of glutamate to bind and react through the ammonia release step. However, the proline profile shows that XH^+ is not required for the proline anion to bind and react. Therefore, we conclude that the presence of XH^+ in the glutamate profile must arise only from its involvement as a general-acid catalyst in hydrolyzing the enzyme-bound α-iminoglutarate to yield ammonia and the enzyme–NADPH–α-ketoglutarate complex and that this group plays no role in the redox step itself.

Group ZH^+. The V_r/K_{Pro} values are unaffected by this group over the pH region 7.5–10.0 as are the V/K values of glutamate and norvaline (Table I). However, the PCA reaction is facilitated by the unprotonated group, Z, while this group is not seen in the α-ketoglutarate profile (Rife & Cleland, 1980). Clearly this group plays no role in the binding of α-ketoglutarate to enzyme–NADPH. The influence of Z on the binding of enzyme–NADPH to α-iminoglutarate and on the reduction of the resulting ternary complex will not be reflected in the ketoglutarate profile. Consequently, it is not clear if there is a similar role for Z in the redox step of the ketoglutarate reaction or if its involvement is a feature present only in the PCA reaction.

[4] We thank Professor W. W. Cleland for suggesting this interpretation.

Scheme II



Chemical Mechanism for Glutamate Dehydrogenase Catalyzed Oxidative Deamination of L-*Glutamate.* Scheme II shows the chemical mechanism that is consistent with both the model studies described here and the ketoglutarate and glutamate profiles (Rife & Cleland, 1980). According to this scheme, glutamate in enzyme–$NADP^+$–glutamate has the amino group in the neutral form while XH^+ forms an ion pair with A^-. The binding of the amino acid to enzyme–$NADP^+$ and of the imino acid to enzyme–NADPH and the hydride transfer step occur without any influence from the protonation states of XH^+ and A^-. In analogy to chemical reactions (Jencks, 1969) we have indicated the nucleophilic attack by water on the bound imino acid as being catalyzed by A^-. The subsequent isomerization step leading to a net transfer of a proton from XH^+ to the amino group is catalyzed by the enzyme presumably through its hydrogen bonding network. Consistent with the suggestion that the hydrolysis of the imino acid is catalyzed by XH^+, we have shown in the scheme that the hydroxyl proton from the conjugate acid of the carbinolamine is abstracted by the unprotonated group X (Jencks, 1969). The resulting bound ketoglutarate is shown hydrogen bonded to XH^+ and HA.

The involvement of XH^+ and A^- shown in the scheme is kinetically equivalent to the participation by X and HA. Consequently, the hydrolysis of the bound imino acid could instead be catalyzed by these neutral enzyme groups as Rife & Cleland (1980) have proposed. However, the model studies reported here require that the protonation states of X and the amino group of glutamate in the enzyme–$NADP^+$–glutamate complex be reversed in their scheme.

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Registry No. PCA, 2139-03-9; NADPH, 53-57-6; $NADP^+$, 53-59-8; L-proline, 147-85-3; glutamate dehydrogenase, 9029-12-3.

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