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Determination of the Chemical Mechanism of Glutamate Dehydrogenase from pH Studies†

James E. Rife and W. W. Cleland*

ABSTRACT: The pH profiles for binding of keto acids and analogues to E–NADPH show that an enzyme group (possibly carboxyl) with a pK of 5 has to be protonated for binding of any molecule, while a group (possibly lysine) with a pK of 7.8 must be protonated to bind the 5-carboxyl of dicarboxylic acids and unprotonated to bind monocarboxylic acids. Changing the 1-carboxyl to a hydroxymethyl group, as in glycolylglycine, does not alter the pH profile, and only weakens binding by a factor of 10, showing that the 1-carboxyl is not bound to lysine or arginine. The V/K profile for ammonia shows that the neutral molecule is the substrate. V/K profiles for glutamate and norvaline show that a group (possibly lysine) with a pK of 7.6–8 must be unprotonated for activity, that the amino acid reacts with its amino group protonated, and that a group (presumably the same lysine involved in binding seen in the reverse reaction) with a pK of 8.7–9.3 must be protonated to bind glutamate, and unprotonated to bind norvaline. We propose that the chemical mechanism of glutamate dehydrogenase involves the attack of neutral ammonia on the ketoglutarate of E–NADPH–ketoglutarate to give a carbinolamine, with the proton for the hydroxyl group coming from the catalytic lysine. After transfer of a proton from N to O of the carbinolamine (possibly catalyzed by the catalytic carboxyl), elimination of water gives iminoglutarate, which is reduced to glutamate by NADPH. The amino group of glutamate is protonated by the catalytic carboxyl to complete the reaction.

The first chemical mechanism proposed for glutamate dehydrogenase (EC 1.4.1.3) was the oxidation of glutamate to α-iminoglutarate, which then decomposed nonenzymatically to α-ketoglutarate and ammonia (von Euler et al., 1938). With the realization that the equilibrium between ketoglutarate and α-iminoglutarate in solution could not support the rate of enzymatic glutamate production, it became apparent that the enzyme directed both the deamination and the dehydrogenation processes (Strecker, 1953). The discovery of two reactive lysines at the active site through modification studies (Rasched et al., 1974; Piszkiewicz et al., 1970; Holbrook & Jeckel, 1969; Colman & Frieden, 1966) prompted the suggestion that ketoglutarate forms a Schiff's base with one of them during the reaction (Smith et al., 1975). The reverse reaction would then proceed with ammonia replacing the lysine in a transimination reaction to form α-iminoglutarate, which would be reduced to glutamate by the coenzyme. However, no direct evidence has been obtained for a Schiff's base between ketoglutarate and lysine. An alternative mechanism involves direct attack by ammonia on ketoglutarate to form a carbinolamine, which forms α-iminoglutarate by loss of water.

In the present work, the chemical mechanism of glutamate dehydrogenase has been studied by examining the pH profiles of V/K for mono- and dicarboxylic keto and amino acid substrates, and of pK_i for inhibitory analogues. The data suggest that the reaction proceeds with direct attack of ammonia on ketoglutarate, and that one lysine acts as an acid–base catalyst, while another functions in binding the 5-carboxyl of dicarboxylic acid substrates.

Materials and Methods

Reagents. Ethanolamine and diethyl oxalate were from Aldrich. Coenzymes, amino and keto acid substrates, and the 2-hydroxyglutarates were from Sigma. Glutaric acid from Sigma was crystallized from hot benzene. Bovine liver glutamate dehydrogenase was obtained from Sigma as a solution in 50% glycerol with pH 7.3 sodium phosphate buffer. This preparation had less than 0.3 μg of NH_4^+ per mg of enzyme. For use, the enzyme was diluted with 0.05 M potassium phosphate buffer, pH 7.0–7.6.

Preparation of Oxalylglycine and Glycolylglycine. Oxalylglycine and glycolylglycine were synthesized by nitrous acid deamination of glycylglycine (Viscontini, 1946). Disodium oxalyglycine was recovered from the reaction mixture as a methanol-insoluble precipitate. This product was washed successively with ethanol and diethyl ether and then recrystallized from H_2O with methanol. Glycolylglycine was recovered from the methanol-soluble fraction of the reaction mixture as the calcium salt. Calcium glycolylglycine was passed through a column of Dowex-1-Cl. After the calcium was washed off the column, the free acid of glycolylglycine was recovered by eluting the column with 0.05 M HCl and evaporating the first acid fractions from the column.

Preparation of N-Oxalylethanolamine. *N*-Oxalylethanolamine was prepared by treatment of ethanolamine with diethyl

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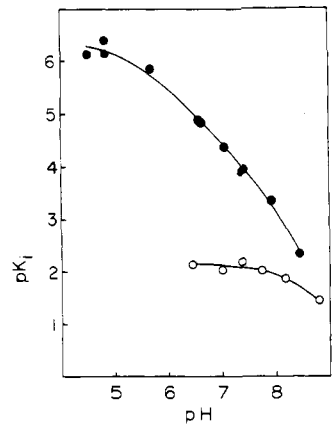


FIGURE 1: pH dependence of the K_is of α-ketoglutarate and α-ketovalerate. K_is were determined at different pHs as described in the text. Ketoglutarate assays (closed circles) had the following composition: (above pH 6) 2.5 mM $(NH_4)_2SO_4$, 0.2 mM NADPH, 0.5 mg/mL bovine serum albumin (BSA), 0.17 unit of glutamate dehydrogenase, and 45 mM phosphate, Hepes, and Tris; ketoglutarate was varied between 0.01 and 0.10 mM below pH 7.5, and between 0.1 and 1.0 mM above pH 7.5; (below pH 6) 2.5 mM $(NH_4)_2SO_4$, 0.02 mM NADPH, between 2.5 and 12.5 units of glutamate dehydrogenase, and 100 mM acetate and 2-(*N*-morpholino)ethanesulfonic acid (Mes); ketoglutarate was varied between 1.0 and 10.0 μM. For the assays below pH 6, 6.0-mL cuvettes with 10.0-cm light paths were used, and the observed K_is were corrected to 0.2 mM NADPH. The curve is a fit to eq 8 with pKs of 5.15 ± 0.06 and 7.82 ± 0.07. Ketovalerate assays (open circles) had the following composition: 25 mM $(NH_4)_2SO_4$, 0.1 mM NADPH, 0.5 mg/mL BSA, 0.5 unit of glutamate dehydrogenase, 100 mM phosphate, Hepes, and Tris, and ketovalerate varied between 2.5 and 25.0 mM. The curve is a fit to eq 5 with a calculated pK of 8.21 ± 0.04.

oxalate (Hope & Horncastle, 1967). The product was recovered from methanol as the barium salt. By passing this barium salt through a column of Dowex-50-H^+, the free acid of *N*-oxalylethanolamine was recovered. The identitiy of the product was confirmed through its NMR spectrum and its melting point of 134–136 °C compared to the reported value of 133–134 °C.

pH Dependence of the Glutamate Dehydrogenase Reaction. The pH dependence of the kinetic parameters was determined by repeating substrate saturation or competitive inhibition experiments in buffers of different pH. To avoid inconsistencies from changing buffers, buffers were prepared containing equal concentrations of several buffering agents and were titrated to the desired pHs. Thus, for a given experiment all reaction mixtures had identical buffer compositions but different ionic strengths. Initial velocities were determined by monitoring the appearance or disappearance of NADPH through its absorbance at 340 nm. All reaction mixtures had a 3.0-mL volume and a 1.0-cm light path. Reactions were started with the addition of enzyme. More detailed descriptions of experimental methods and conditions are given in the figure captions.

Statistical Analysis of the Data. Values of kinetic constants were determined by fitting initial velocity and concentration data to the appropriate rate equations by the least-squares method using the Fortran programs of Cleland (1967). Substrate saturation curves were fit to eq 1, while competitive

$$v = VA/(K + A) \quad (1)$$

inhibition data were fit to eq 2. Intersecting initial velocity

$$v = VA/[K(1 + I/K_i) + A] \quad (2)$$

patterns were fit to eq 3.

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (3)$$

pK values were obtained by fitting the pH profiles to one of the following equations (eq 4–11).

$$\log y = \log [C/(1 + H/K)] \quad (4)$$

$$\log y = \log [C/(1 + K/H)] \quad (5)$$

$$\log y = \log [C/(1 + H/K_2 + K_3/H + H^2/K_1K_2)] \quad (6)$$

$$\log y = \log [C/(1 + H/K_1 + K_2/H + K_2K_3/H^2)] \quad (7)$$

$$\log y = \log [C/(1 + K_1/H + K_1K_2/H^2)] \quad (8)$$

$$\log y = \log [CH^2/(H + K)] \quad (9)$$

$$\log y = \log [C/(1 + H/K_1 + K_2/H)] \quad (10)$$

$$\log y = \log [C/(1 + H/K_1 + H^2/K_1K_2)] \quad (11)$$

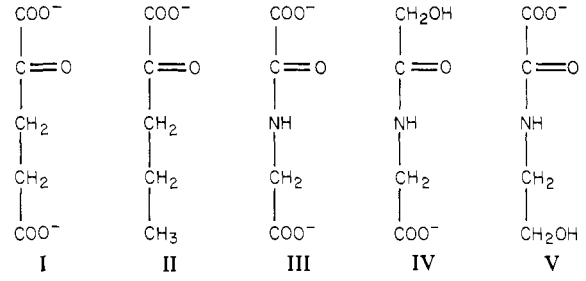
Determination of ΔH_{ion} Values. The enthalpies of ionization of enzymatic residues were determined by obtaining pH profiles at different temperatures and then fitting the observed pKs to equation 12. To correct for changes in the buffer pKs

$$pK = -(\Delta H_{ion}/2.3RT) + C \quad (12)$$

with temperature, the pHs of the reaction mixtures were measured directly at the reaction temperature.

Results

pH Dependence of Ketoglutarate and Ketovalerate Dissociation Constants from the E–NADPH Complex. K_i values for the keto acids were determined as their apparent K_ms at low levels of ammonia, but with saturating NADPH. In Figure 1, the plot of pK_i for ketoglutarate (I) vs. pH has a slope



of 0 below pH 5, a slope of −1 between pH 5 and 7.8, and a slope of −2 at higher pHs. This curve indicates that groups with pKs of 5.2 and 7.8 must both be protonated for E–NADPH to bind ketoglutarate. A similar profile was seen with NADH as the nucleotide. In contrast, the plot for ketovalerate (II) has a plateau at low pH and decreases with a slope of −1 above a pK of 8.2. The pK_i profile for ketobutyrate was similar to that of ketovalerate. Thus, for ketovalerate to bind to E–NADPH, one of the groups seen in the ketoglutarate profile must be protonated, while the other must be unprotonated.[1] The fact that ketoglutarate binds much more tightly than ketovalerate suggests that it is the group with a pK of 8.2 that must be unprotonated for ketovalerate to bind, rather than the group with a pK of 5.2.[2] Thus, the group with

[1] A more remote possibility is that the protonation state of the group with pK = 5.2 is important for binding of dicarboxylic acids, but not for monocarboxylic ones, and thus that the group with a pK of 8.2 must be protonated for binding of all molecules. It is difficult to see a reasonable mechanism for this, and the requirement for a group with a pK around 9 in E–$NADP^+$ to be protonated for reaction of glutamate, but unprotonated for reaction of norvaline, argues against such an interpretation.

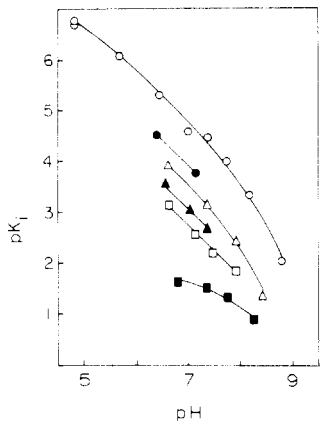


FIGURE 2: pK_i profiles for competitive inhibitors of ketoglutarate and ketovalerate. (○) Oxalylglycine: The assays contained 0.1 mM NADPH, 25 mM $(NH_4)_2SO_4$, 100 mM phosphate, Hepes, and Tris, 0.5 mg/mL BSA, 2.5–25 mM ketovalerate, and 0–0.68 mM oxalylglycine. The curve is a fit to eq 8 with pKs of 4.70 ± 0.10 and 7.98 ± 0.06. (●) Glycolylglycine: Assays contained 2.5 mM $(NH_4)_2SO_4$, 0.2 mM NADPH, buffers and BSA as above, 10–100 μM ketoglutarate, and 0–0.8 mM glycolylglycine. (△) L-2-Hydroxyglutarate: The conditions were identical with those in Figure 1 for ketoglutarate with the addition of 0–20 mM inhibitor at the highest pH and 0–2 mM for the others. The curve is a fit to eq 9 with a pK of 7.79 ± 0.08. (▲) D-2-Hydroxyglutarate: The conditions were the same with 0–2 mM inhibitor. (□) Glutarate: The conditions were the same as with glycolylglycine with 5–50 μM ketoglutarate and 0–4.0 mM glutarate for the two low pHs, and 50–500 μM ketoglutarate and 0–10 mM glutarate for the high pHs. (■) N-Oxalylethanolamine: The conditions were similar, except the buffers were 100 mM phosphate, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes), and Tris, while ketoglutarage was 50–500 μM for the highest pH, and 10–100 μM for the others, and the inhibitor was 0–30 mM. The curve is a fit to eq 5 with a pK of 7.50 ± 0.05. Lines with a slope of −1 are drawn through the data for glycolylglycine, D-hydroxyglutarate, and glutarate.

pK = 5.2 has to be protonated for either a mono- or dicarboxylic keto acid to bind.

pH Dependence of the Inhibition by Keto Acid Analogues. In Figure 2, the pK_i profiles for several competitive inhibitors of keto acid substrates are compared. The pK_i profiles of oxalylglycine (III) and ketoglutarate are identical, confirming that binding of a dicarboxylic keto acid requires two enzyme groups to be protonated in the E–NADPH complex. Since the K_is of the 2-hydroxyglutarates and glutarate decrease with decreasing pH, reduction or elimination of the 2-keto group does not relieve the requirement for protonation of the group with a pK around 5, and therefore that group has a function other than (or in addition to) hydrogen bonding to the keto oxygen. The data with glycolylglycine (IV) suggest that it, too, requires protonation of the group with pK around 5, and thus that this group is not the 1-carboxyl of the substrate or inhibitor. It is interesting that, of the inhibitors compared here, glycolylglycine has the second highest affinity; these data show that the 1-carboxyl is not bound to lysine or arginine on the enzyme, but rather by hydrogen bonds to neutral groups, or to NH groups of amides of the backbone or glutamine or asparagine side chains which carry a partial positive charge. The observation by Caughey et al. (1957) that the inhibitors

[2] That is, it is the reverse protonation state of the enzyme (protonated group with pK = 5.2; ionized group with pK = 8.2) that binds monocarboxylic keto acids. Since only 0.1% of the enzyme has this protonation state at neutral pH (because of the 3 pH unit separation of the pKs, the rest having the low pK group ionized and the high pK group protonated), the K_i value for monocarboxylic keto acids is expected to be at least three orders of magnitude higher than that for dicarboxylic acids. The values in Table I show this to be the case.

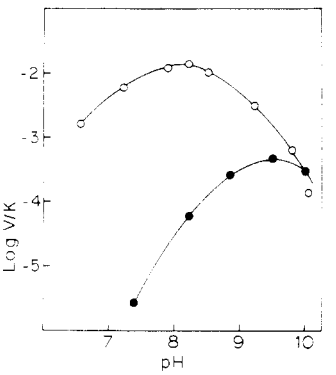


FIGURE 3: pH dependence of the V/Ks of glutamate and norvaline. The glutamate assays (open circles) contained 1.0 mM $NADP^+$, 100 mM phosphate, Hepes, Tris, and CHES, and 0.67 unit of glutamate dehydrogenase. Glutamate was varied between 0.5 and 5.0 mM. The curve is a fit to eq 7 with pK_1 = 7.63 ± 0.08, pK_2 = 8.7 ± 0.2, and pK_3 = 9.3 ± 0.3. For norvaline (closed circles), the assays contained 1.0 mM $NADP^+$, 100 mM phosphate, Tris, and glycine, and 0.5 unit of glutamate dehydrogenase. Norvaline was varied between 10 and 100 mM. The curve is a fit to eq 6 with pK_1 = 8.05 ± 0.08, pK_2 = 9.26 ± 0.06, and pK_3 = 9.60 ± 0.06. The units of V/K are min^{-1}.

Table I: Comparison of the K_is of Keto Acid Substrates and Inhibitors and the pKs Seen in Their pK_i Profiles

substrate or inhibitor	K_i at pH 6.5 (mM)	pKs obsd in pH profile	profile described by eq
α-ketoglutarate	0.0027–0.0110[b]	5.15 ± 0.06, 8.00 ± 0.17[a]	8
oxalylglycine	0.0039–0.0051[b]	4.7 ± 0.1, 8.15 ± 0.18[a]	8
glycolylglycine	0.040	[c]	[c]
L-2-hydroxyglutarate	0.0805	7.79 ± 0.08	9
D-2-hydroxyglutarate	0.27	[c]	[c]
glutarate	0.6–2.1[b]	[c]	[c]
α-ketovalerate	3.1–7.3[b]	7.82 ± 0.24[a]	5
α-ketobutyrate	6.83	7.57 ± 0.05	5
N-oxalylethanolamine	20	7.50 ± 0.05	5

[a] These pKs are the averages and standard errors of several experiments. The other pKs and standard errors were statistically determined from single experiments. [b] Range observed in different experiments. [c] Insufficient data to determine the pK; the K_i increases a factor of 10 per pH unit over the pH range investigated.

Table II: Temperature Dependence of the Glutamate V/K Profile[a]

expt	temp (°C)	optimum pH $[(pK_1 + pK_2)/2]$	ΔH_{ion} (kcal/mol)
I	15.0	8.37 ± 0.08	
	25.1	8.05 ± 0.11	11.8 ± 0.5
	32.1	7.88 ± 0.04	
II	15.1	8.43 ± 0.09	
	24.4	8.06 ± 0.04	14.6 ± 0.7
	32.0	7.82 ± 0.05	

[a] The V/K of glutamate was determined at each temperature, and apparent values for pK_1 and pK_2 were determined by fitting the data to eq 10. The average ΔH_{ion} of groups 1 and 2 was determined by fitting the average of pK_1 and pK_2 to eq 12. The assays contained 100 mM each of phosphate, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), Tris, and 2-(cyclohexylamino)ethanesulfonic acid (CHES) and 0.67 unit of glutamate dehydrogenase. Glutamate was varied between 0.5 and 5.0 mM. $NADP^+$ was 2.0 mM in experiment I and 1.0 mM in experiment II.

m-iodo- and m-bromobenzoate are as effective as the inhibitor isophthalate suggests that both carboxyls of glutamate are not bound to positively charged groups. Finally, the pK_i profile

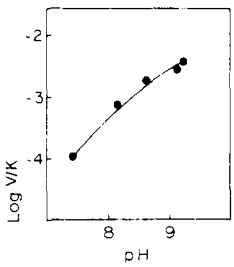


FIGURE 4: pH dependence of $V/K_{ammonia}$. Assays contained 0.3 mM NADPH, 100 mM phosphate, Hepes, Tris, and CHES, 1.0 mg/mL BSA, 1.0 mM ADP, 0.5 unit of glutamate dehydrogenase, ketovalerate varied between 3 and 30 mM, and $(NH_4)_2SO_4$ varied between 5 and 50 mM. The initial velocity pattern at each pH with saturating NADPH and ammonia and ketovalerate as variable substrates was fitted to eq 3, and the resulting V/K values for ammonia were fitted to eq 4, giving a pK of 9.05 ± 0.07. The curve assumes that free ammonia with a pK of 9.25 is the active species. The units of V/K are min^{-1}.

of *N*-oxalylethanolamine (V) is similar to that of ketovalerate. Since ketovalerate and oxalylethanolamine do not have 5-carboxyl groups, it appears that the enzyme group with a pK around 8 has to be protonated to bind a molecule with a 5-carboxyl group, but neutral to allow binding of monocarboxylic substrates or inhibitors. Thus, the 5-carboxyl of ketoglutarate, unlike the 1-carboxyl, is bound to a positively charged group.

The relative affinities of glutamate dehydrogenase for all of the analogues used are shown in Table I.

Characterization of the Groups in the Keto Acid Profiles. When the pK_i profile for ketoglutarate was repeated at several temperatures, a ΔH_{ion} of 18 ± 1 kcal/mol was measured for the group with a pK of 7.9. In the pK_i profiles for ketovalerate, where this pK is better defined, a ΔH_{ion} of 11 ± 2 kcal/mol was seen. Such ΔH_{ion} values are consistent with this group being a lysine, but are at least twice the value expected for histidine. The group with a pK of 7.9 in the pK_i profile of ketoglutarate was further characterized by the effect of dimethyl sulfoxide on its pK. With cationic buffers, dimethyl sulfoxide had no effect on the apparent pK, while in neutral acid buffer, it caused the apparent pK to decrease slightly. Such behavior is consistent with this group being a cationic acid, such as lysine. The nature of the group with a pK of 5.2 has not been determined, but its low pK suggests it is a carboxyl group, and this assignment makes more sense in the chemical mechanism than for it to be a cationic acid (see Discussion).

pH Dependence of V/K for Glutamate and Norvaline. As shown in Figure 3, the pH dependence of $V/K_{glutamate}$ shows that two groups with pKs of 8.7 and 9.3 must be protonated for glutamate to bind and react, while a third group with a pK of 7.6 must be unprotonated. In contrast, the norvaline profile has two acid side and one base side ionizable groups.[3] Clearly, the group with a pK of 8.7–9.3, which must be protonated for glutamate to bind, must be unprotonated for norvaline to bind.[3] As this requirement for opposite protonation is identical with that seen with ketoglutarate and ketovalerate, this group is very likely the same lysine seen with the keto acids, and has the same function, namely, to bind the 5-carboxyl of the substrate.

The group on the basi side of the profiles with a pK of 9.3–9.6 is probably the α-amino group of the substrates, which has a pK of 9.67 in glutamate and 9.72 in norvaline (Greenstein & Winitz, 1961). Thus, the enzyme binds substrate molecules which have the amino group protonated. The group on the acid side with a pK of 7.6–8 must be unprotonated for binding of either glutamate or norvaline.[3] This group probably does not appear in the keto acid profiles because its pK is much higher when NADPH rather than $NADP^+$ is present, and it is protonated for reaction of keto acids (as we will show shortly, the enzyme must supply two protons for elimination of the carbonyl oxygen of ketoglutarate as water; one presumably comes from this group, and the other from the group with pK = 5 in the keto acid profiles). Finally, the group with a pK around 5 which must be protonated for binding of keto acids does not affect the amino acid profiles, so that binding of the amino acid substrates occurs when this group is unprotonated.

Characterization of the Groups in the Amino Acid V/K Profiles. When glutamate was the substrate, ΔH_{ion} values of 16.0 ± 1.5, 8.8 ± 0.5, and 18 ± 4 kcal/mol were obtained for the pKs at 7.6, 8.7, and 9.3, respectively. Interpretation of these values is uncertain because the pKs are so close together in the glutamate profiles. If the pKs are less than 2 pH units apart in such curves, the calculation of the pKs becomes very dependent on the shape of the top of the curve, and is open to considerable error (Cleland, 1977). However, the pH optimum of the curve does accurately mark the median between the first two pKs, as can be seen in Table II. The values of 12–15 kcal/mol for the enthalpy of ionization of the average of pK_1 and pK_2 suggest that both groups have high enthalpies of ionization and therefore could be lysines. In the norvaline V/K profile, it is possible to obtain a better defined pK for the second group. From these data a ΔH_{ion} of 13 ± 3 kcal/mol was measured. We interpret these results to mean that amino acid binding is dependent on a lysine with a pK between 7.6 and 8 being unprotonated, a second lysine with a pK between 8.7 and 9.3 being protonated for glutamate and unprotonated for norvaline, and the α-amino group of the substrate being protonated.

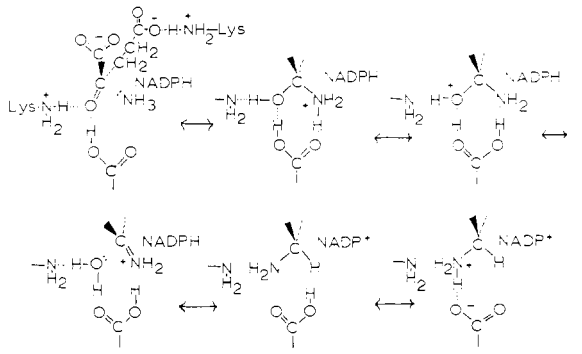
pH Dependence of $V/K_{ammonia}$. The pH profile of the V/K for ammonia in the presence of saturating NADPH and ketovalerate (Figure 4) shows a requirement for a group with a pK of 9.05 to be unprotonated. Since this pK is very close to that of ammonia (9.25), it appears that free ammonia rather than ammonium ion is the active substrate. Since the amino group of the amino acid substrate which must be protonated for combination with the enzyme contains the same number of protons as NH_3, the enzyme must thus donate two protons to allow the elimination of the carbonyl oxygen of the keto acid as water, in agreement with the assumption made above.

Discussion

The results presented here strongly suggest that (1) the amino group of the amino acid substrate must be protonated; (2) a group with a pK of 7.8 in E–NADPH and 8.7–9.3 in E–$NADP^+$, and which is a cationic acid and has an enthalpy of ionization characteristic of lysine, must be protonated for binding of dicarboxylic acids but unprotonated for binding of monocarboxlyic acids, and thus participates in binding the 5-carboxyl group; (3) the 1-carboxyl of the substrate is not bound by ionic bonds to lysine or arginine; (4) a group on the enzyme with a pK of 7.6–8 in E–$NADP^+$ but at least 10.5 in E–NADPH, and an enthalpy of ionization characteristic of

[3] In complex profiles like those in Figure 3, it is clear how many pKs are present, and whether the groups involved must be protonated or unprotonated, but it is more difficult to establish exact values for the pKs, even though least-squares fits give numbers and standard errors. Thus, it is clear that the pK which appears to vary from 7.6 to 8 is for a single group on the enzyme, as is the pK which varies from 8.7 to 9.3. As shown in Table II, the average of two closely spaced pK values for groups, one of which must be protonated and the other unprotonated, can be determined with greater precision than the individual pK values themselves.

Scheme I



lysine, must be unprotonated for combination of glutamate, but protonated for reaction of keto acids; and (5) a group with a pK of 5.2 in E–NADPH has to be protonated for binding of keto acids, but unprotonated for combination of amino acids.

It could be argued that the identification of two of the enzyme groups critical for binding and reaction of substrates as lysines solely on the basis of enthalpies of ionization (although one is also known to be a cationic acid) is unwarranted. However, amino acid modification studies have shown that glutamate dehydrogenase has two very reactive lysines, which are residues 126 (Colman & Frieden, 1966; Holbrook & Jeckel, 1969; Piszkiewicz et al., 1970) and 27 (Rasched et al., 1974). Both of these lysines have been implicated in substrate binding (Brown et al., 1973; Rasched et al., 1974) and have unusually low pKs. Modification studies have indicated that lysine-126 has a pK of 7.7–7.8 (Piszkiewicz & Smith, 1971), and lysine-27 has a pK of 8.2 (Rasched et al., 1974). These pKs are similar to the pKs observed for the lysines in this study. Other workers who have examined the pH dependence of the glutamate dehydrogenase reaction have also noticed groups that ionize with pKs in the vicinity of 8.0 (Rogers, 1971; Deppert et al., 1973). It is interesting that both of these lysines have been conserved in all the $NADP^+$ utilizing glutamate dehydrogenases whose primary structures are known (Smith et al., 1975), and no other specific residues have been conclusively linked with the enzyme's active site through modification studies. As a result we believe the assignment of the two groups we see in the pH profiles as lysine-27 and -126 is reasonable.

Scheme I shows a chemical mechanism for glutamate dehydrogenase which is consistent with all available data. The 5-carboxyl of ketoglutarate is shown hydrogen bonding to lysine, but the 1-carboxyl is hydrogen bonded to formally neutral groups. The catalytic lysine is shown hydrogen bonding to the carbonyl oxygen of ketoglutarate, as is the putative carboxyl group with a pK of 5, which must be protonated for binding of keto acids and their analogues. Possibly the catalytic lysine and this carboxyl bind to each other when the carboxyl is ionized, thus altering the geometry of the active site to prevent substrate or analogue binding. The catalytic role of this carboxyl is speculative, but since the proton transfer between the nitrogen and oxygen of the carbinolamine must be catalyzed in some way, and the protonated amino group of glutamate must be deprotonated before hydride transfer, the proposed mechanism is certainly reasonable.

In this scheme, the reverse reaction begins with free ammonia attacking the keto carbon as the oxygen is protonated by the catalytic lysine. One proton must be transferred from the amino to the hydroxyl group of the resulting carbinolamine before expulsion of water to form iminoglutarate, and this process is shown being catalyzed by the carboxyl group.[4] Reduction of iminoglutarate by NADPH produces L-glutamate with a neutral amino group, which is protonated by the carboxyl group to yield the form of glutamate shown to be the substrate. The ionic bond between the protonated amino group and the catalytic carboxyl may explain why glutamate binds to free enzyme and the kinetic mechanism of the forward reaction is random (Rife & Cleland, 1980). Note how in successive steps in Scheme I the positive charge in the active site oscillates between the left and right sides, alternatively being on the catalytic lysine, the carbinolamine nitrogen, the carbinolamine oxygen (or catalytic lysine), the imino nitrogen, and the nicotinamide ring of $NADP^+$. This migrating electron deficiency certainly plays an important role in the catalytic process.

The stereochemistry of the carbinolamine intermediate in Scheme I is that suggested by the stronger binding of L- rather than D-2-hydroxyglutarate. In this model, ketoglutarate and iminoglutarate are not adsorbed in the same way, with the carbonyl oxygen of ketoglutarate being in contact with the catalytic lysine, while the nitrogen of iminoglutarate is farther away, with a water molecule in between. This arrangement would explain why ketoglutarate is not reduced to hydroxyglutarate, but why iminoglutarate is close enogh to NADPH for hydride transfer between them. However, the alternate mechanism in which attack of ammonia is from the side opposite to NADPH, and the carbinolamine has the opposite stereochemistry, cannot be ruled out, and is still a possibility.

We should also comment on the fact that groups seen in the pK_i profiles for keto acids have different pKs in the V/K profiles for the amino acids. Thus, the lysine which binds the 5-carboxyl of dicarboxylic substrates has a pK of 7.9 in E–NADPH, and of 8.7–9.3 in $E–NADP^+$. The lysine which acts as an acid–base catalyst has a pK of 7.6–8 in $E–NADP^+$, but a pK above 10 in E–NADPH. The normal pK of this group is probably high, but when the oxidized coenzyme with its positively charged nicotinamide ring binds, the pK is displaced to the value seen. This lysine will in turn affect the pK of the one which binds the 5-carboxyl of substrates, lowering its pK from the value seen in E–NADP (at which pH the catalytic lysine is not protonated in E–NADP) to that seen in E–NADPH (at which pH the catalytic lysine is protonated in E–NADPH). These changes are consistent with the catalytic lysine lying very close to the nucleotide, and the binding lysine being somewhat farther away. We have no way at the moment of determining which lysine is 27 and which is 126.

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[4] This is somewhat speculative, as any hydrogen bonding network involving one or more hydroxyl groups of serine, threonine, or tyrosine could also accomplish this catalysis. We note, however, that the proton transfer must be catalyzed by the enzyme.

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Role of Magnesium Adenosine 5′-Triphosphate in the Hydrogen Evolution Reaction Catalyzed by Nitrogenase from *Azotobacter vinelandii*†

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ABSTRACT: We have investigated the role of MgATP in the reaction catalyzed by nitrogenase from *Azotobacter vinelandii*. There is a rapid burst of ATP hydrolysis in the pre-steady-state reaction that occurs on the same time scale as the electron transfer from dinitrogenase reductase to dinitrogenase. This burst corresponds to two ATP's hydrolyzed per electron transferred between the two proteins. Two MgATP molecules are bound to dinitrogenase reductase with dissociation constants of 430 μM and 220 μM. Investigation of the effect of MgATP concentration on the pre-steady-state kinetics of electron transfer from dinitrogenase reductase to dinitrogenase showed that there are two MgATP's required for this reaction, and the K_m values are 220 μM and 970 μM. These values are similar to the dissociation constants for MgATP from dinitrogenase reductase and indicate that electron transfer between the two proteins is substantially slower than the binding and dissociation of MgATP from dinitrogenase reductase. The K_m values for MgATP in steady-state H_2 evolution were 390 μM and 30 μM. The decrease in the value of the second K_m indicates that a slow, irreversible step occurs after the electron transfer from dinitrogenase reductase to dinitrogenase. It is possible to predict quantitatively the steady-state kinetics from the pre-steady-state kinetics, and this shows that the MgATP dependence of electron transfer is sufficient to account for effects of MgATP concentration on the steady-state H_2 evolution catalyzed by nitrogenase. The hydrolysis of two ATP molecules when an electron is transferred between the two proteins of the nitrogenase system is sufficient to account for all of the ATP hydrolysis occurring in the steady-state reaction. The simplified scheme proposed to account for the MgATP dependency of the nitrogenase reaction indicates that the only role of MgATP is in support of the electron transfer from dinitrogenase reductase to dinitrogenase.

The nitrogenase enzyme system is responsible for the biological reduction of N_2 to NH_3 in an MgATP-requiring reaction. The enzyme system also catalyzes MgATP-dependent H_2 evolution and the MgATP-dependent reduction of acetylene to ethylene. The enzyme system consists of two proteins, dinitrogenase[1] (MoFe protein) and dinitrogenase reductase (Fe protein) (Zumft, 1976). Dinitrogenase carries the substrate reducing site, and dinitrogenase reductase serves as a specific reductase for dinitrogenase (Hageman & Burris, 1978a). The enzymology of nitrogenase has been reviewed frequently (Orme-Johnson et al., 1977; Winter & Burris, 1976; Zumft, 1976), and this paper will concentrate on the role of MgATP [also see a recent review by Ljones (1979)].

Although the nitrogenase reaction of physiological importance is the reduction of N_2, the reduction of protons to H_2 is a more convenient reaction for studies of electron flow through nitrogenase:

$$2H^+ + 2e^- + n\text{MgATP} \rightarrow H_2 + n\text{MgADP} + nP_i$$

The amount of MgATP hydrolyzed in the reaction is variable and ranges from a minimum of 4 ATP per 2 electrons to greater than 20 (Ljones & Burris, 1972), with no evidence for an upper limit. Dinitrogenase reductase contains a single [4Fe–4S] cluster[2] (Orme-Johnson & Davis, 1977), and it donates a single electron per [4Fe–4S] cluster to dinitrogenase in the physiological reaction (Ljones & Burris, 1978a). A molecule of dinitrogenase reductase binds two MgATP molecules (Tso & Burris, 1973), and the binding of both of these MgATP molecules is required to induce a conformational change in the Fe protein (Walker & Mortenson, 1973; Ljones & Burris, 1978b; Zumft et al., 1973). The binding of two MgATP's and the donation of one electron by dinitrogenase reductase agree nicely with the minimum hydrolysis of four ATP per two electrons.

MgATP is required to support electron transfer from dinitrogenase reductase to dinitrogenase (Orme-Johnson et al., 1972; Zumft, 1976). Eady et al. (1978) have shown that MgATP is hydrolyzed with a time course identical with that of the pre-steady-state electron transfer from dinitrogenase reductase to dinitrogenase of *Klebsiella pneumoniae*. They analyzed the stoichiometry of this burst of MgATP hydrolysis

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[1] The nomenclature used in this paper is modified somewhat from that suggested by Hageman & Burris (1978a). The MoFe protein is designated dinitrogenase and the Fe protein is designated dinitrogenase reductase to express the activity of the components of the complete system that is designated nitrogenase.

[2] Abbreviations used: [4Fe–4S], a four-iron- and four-sulfur-containing cluster of the ferredoxin type; BPS, bathophenanthrolinedisulfonate.

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