α-Ketoglutaric Acid: Solution Structure and the Active Form for Reductive Amination by Bovine Liver Glutamate Dehydrogenase†

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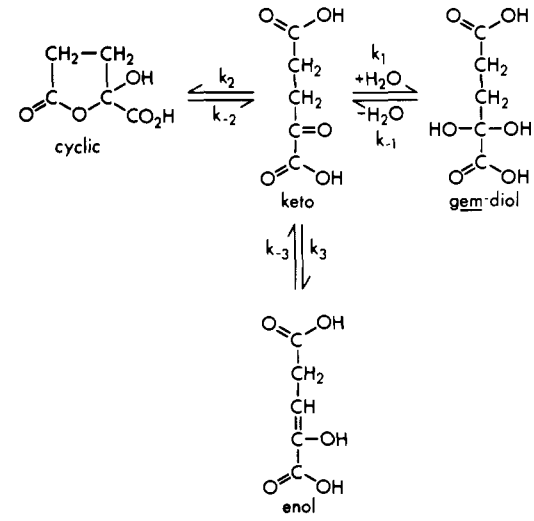
ABSTRACT: A study of the various solution forms of α-ketoglutaric acid using UV absorption spectrophotometry and ^{13}C NMR spectroscopy shows that at neutral pH α-ketoglutarate exists predominantly as the keto form with about 7% hydrated form (*gem*-diol) and a small amount of cyclic form. Protonation of the γ-carboxylate group increases the amount of cyclic form to 20% with very little increase in the amount of *gem*-diol. Protonation of the α-carboxylate increases the amount of cyclic form to 30% and the amount of *gem*-diol to 35%. The pH and temperature dependence of the ^{13}C NMR line widths indicated that the interconversion of keto and cyclic forms is extremely rapid. The rate of interconversion of keto and *gem*-diol forms was studied spectrophotometrically at various temperatures, buffers, and pH values and by varying the total concentration of α-ketoglutarate. The hydration reaction of α-ketoglutarate is not catalyzed by bovine liver glutamate dehydrogenase (E) or by the E–NADPH complex. The enzyme uses the keto form of α-ketoglutarate as the substrate. The *gem*-diol form is not itself a substrate but becomes converted to the keto form with a half-life of about 0.3 min at 15 °C in 0.1 M potassium phosphate buffer (pH 7.6).

Carbonyl compounds can exist in a number of tautomeric or hydrated forms (Bell, 1966). A specific form of a carbonyl compound may be a substrate for one enzyme while inhibiting another enzyme. In some cases such as in oxaloacetate, the tautomerism may be catalyzed by a tautomerase (Annett & Kosicki, 1969). Examples of enzymes have specificity for the keto form of carbonyl compound include lactate dehydrogenase (Tienhaara & Meany, 1973; Loewus et al., 1955), malate dehydrogenase (Loewus et al., 1955; Annett & Kosicki, 1969), citrate synthase (Englard, 1959; Annett & Kosicki, 1969), citrate lyase (Tate & Datta, 1964), malic-lactic transhydrogenase (Dolin, 1968), phosphoenolpyruvate carboxylase (Tchen et al., 1955) phosphoenolpyruvate carboxykinase (Graves et al., 1956), triosephosphate isomerase, aldolase, α-glycerophosphate dehydrogenase (Reynolds et al., 1971), oxalacetate decarboxylase, and glutamate-oxalacetate transaminase (Annett & Kosicki, 1969).

α-Ketoglutaric acid, an important metabolite and a product (or substrate) of several enzymatic reactions can exist in several forms in solution. Some of the forms are shown in Scheme I. Cooper & Redfield (1975) have made a careful study of the proton NMR[1] spectrum of α-ketoglutaric acid and several other α-keto acids and concluded that α-ketoglutaric acid exists as a mixture of 16% cyclic form, 53% *gem*-diol, and 31% keto forms at pH 0.5.

We have been studying the mechanism of the glutamate dehydrogenase reaction with an emphasis on the role of the keto group of α-ketoglutarate using cryoenzymological techniques (Johnson et al., 1981) and carbonyl oxygen exchange kinetic methods. These studies required a thorough examination of the nature and the amount of the various species of α-ketoglutaric acid in neutral and acidic solutions and the rate at which these species interconvert under various conditions. This paper describes such studies done by using ^{13}C nuclear magnetic resonance and UV absorption spectrophotometry and other studies directed to finding out which form of α-ketoglutarate is utilized by glutamic dehydrogenase, the enzyme that catalyzes the reversible reductive amination of α-ketoglutarate and ammonia to L-glutamate.

Scheme I



Experimental Procedures

Materials. α-Ketoglutaric acid, α-ketovaleric acid, γ-ketovaleric acid (levulinic acid), and NADPH were purchased from Sigma Chemical Co. Sodium pyruvate (99.9% pure) was obtained from Mann Research Laboratories. Bovine liver glutamate dehydrogenase was obtained as an ammonium sulfate suspension from Boehringer Mannheim and treated with Norit A as described elsewhere (Cross & Fisher, 1970). All other reagent-grade chemicals used in this work were obtained from standard sources.

Methods. The concentration of the enzyme was determined by measuring its absorbance at 280 nm and using an ab-

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[1] Abbreviations: GDH and E, glutamate dehydrogenase; *gem*-diol or hydrate, 2,2-dihydroxyglutarate; lactol or cyclic form, 2-hydroxy-5-oxotetrahydrofuran-2-carboxylic acid; NMR, nuclear magnetic resonance; Me_4Si, tetramethylsilane; FID, free induction decay; FT, Fourier transform; NOE, nuclear Overhauser effect.

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sorptivity of 0.97 cm^{-1} mg^{-1} mL (Olsen & Anfinsen, 1952). The absorbance of NADPH at 340 nm (ϵ_{340} = 6300 M^{-1} cm^{-1}) was used to calculate its concentration (McComb et al., 1976). All other concentrations were determined by weight.

Spectroscopic Measurements. Carbon-13 NMR spectra were obtained at 50.32 MHz on a Bruker WP200 NMR spectrometer equipped with an Aspect 2000 computer in the pulsed FT mode. Most of the NMR measurements were done at the ambient probe temperature (29 °C) at 0.5 W of proton decoupling power. Dioxane (0.625% v/v) was used as the internal reference, and all chemical shifts were calculated in ppm downfield from Me_4Si by assuming that the chemical shift of dioxane is 67.40 ppm (Levy & Cargioli, 1972). All samples contained approximately 5% v/v D_2O to serve as an internal field-frequency lock material. The ^{13}C NMR spectra were obtained with a sweep width of 12 000 Hz and a data length of 8192 points for FID. The minimum line width which could be measured accurately was about 6 Hz. This was determined by the number of data points per hertz in the Fourier transformed spectrum (0.34) and the exponential smoothing function (line broadening = 2 Hz) which was applied to all FID's before Fourier transformation. Proton undecoupled ^{13}C NMR spectra with NOE were obtained by gating the decoupler power.

The UV absorption measurements were done by using either a Cary-14 spectrophotometer interfaced to a 8K Varian 620i computer or a Gilford 2000 spectrophotometer equipped with a rapid sampler.

Determination of the Rate Constants for Keto ⇌ gem-Diol Interconversion in α-Ketoglutarate. An aqueous solution of 0.25 M α-ketoglutaric acid was acidified to pH 1.05. At this pH, the solution contains 35% *gem*-diol compared to a solution at neutral pH which contains only about 6% *gem*-diol. The dehydration reaction was initiated by adding 0.1 mL of this solution to 2.4 mL of a buffered solution (pH 7.6) in a thermostated cuvette. An appropriate amount of standardized sodium hydroxide was added to the buffer to counterbalance any decrease in pH. The reaction was followed by monitoring the increase in absorbance at 250 or 320 nm as the concentration of the keto form increases. The observed first-order rate constant, k, for the approach of keto and *gem*-diol concentrations to equilibrium levels was determined by fitting the absorbance (A) vs. time (t) data to

$$A_t = A_\infty + (A_0 - A_\infty)e^{-kt} \quad (1)$$

The observed rate constant is actually the sum of the rate constants for the hydration of the keto form and the dehydration of the *gem*-diol form due to the reversibility of the reaction. The individual rate constants are obtained by determining the equilibrium constant K under the same conditions (see Results and Discussion) and solving the equations $K = k_1/k_{-1}$ and $k = k_1 + k_{-1}$.

The first-order rate constant k for the approach of keto and *gem*-diol concentrations to equilibrium levels during the enzymatic reductive amination reaction was determined by fitting the data to

$$\Delta A_{340} = a + be^{-kt} + ct \quad (2)$$

where a, b, and c are constants and ΔA_{340} is the difference in the coenzyme absorption (at time t) between two reactions, with one initiated by the keto form and the other initiated by the *gem*-diol form of α-ketoglutarate at a fixed total concentration of enzyme, NADPH, NH_4Cl, and α-ketoglutarate in both cases. The first and last terms in eq 2 account for small variations in the temperature and the concentrations of enzyme and α-ketoglutarate.

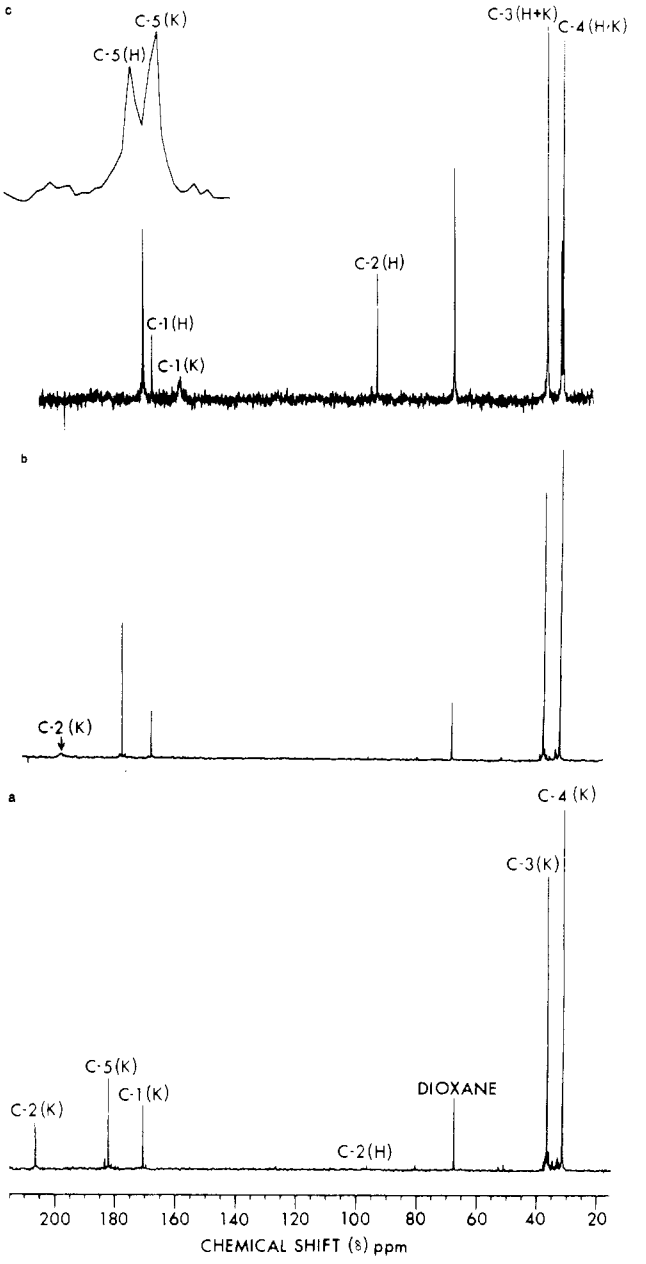

FIGURE 1: Proton decoupled ^{13}C NMR spectrum of 1 M α-ketoglutaric acid at 50.32 MHz (a) at pH 6.08, (b) at pH 4.60, and (c) at pH 0.11. The inset in (c) shows the downfield peaks after 40-fold horizontal expansion. The letter in parentheses following the carbon number shows the nature of the species: K = keto form and H = hydrate or *gem*-diol form.

Results and Discussion

(I) Solution Structure of α-Ketoglutaric Acid. (A) Assignment of ^{13}C NMR and UV Signals. The proton decoupled ^{13}C NMR spectrum of α-ketoglutarate at 29 °C and at pH 6.36 is shown in Figure 1a. Previous studies (Cooper & Redfield, 1975) have shown that at this pH α-ketoglutarate exists predominantly in the keto form with a small amount of *gem*-diol. Thus, the spectrum shown in Figure 1a, which consists of five sharp resonances, was assigned to the keto form. The downfield signal at 206.74 ppm from Me_4Si was assigned to the carbonyl (C-2) carbon by comparison of its chemical shift with the chemical shift of the carbonyl resonance in pyruvic acid and other ketones (Stothers, 1972).

The resonance at 170.65 ppm in the carboxyl region was assigned to C-1 of α-ketoglutarate by comparison with the carboxyl resonance of α-ketovaleric acid, which has a chemical shift of 171.77 ppm at pH 7.4 and 29 °C. The other carboxyl

resonance at 181.95 ppm was similarly assigned to C-5 by comparison with the carboxyl resonance of γ-ketovaleric acid, which occurs at 182.13 ppm from Me_4Si at pH 7.7, and from the fact that the proton undecoupled spectrum of the 181.95-ppm resonance in α-ketoglutarate consists of a five-line multiplet, showing 2-bond and 3-bond coupling to the adjacent methylene protons, while the 170.65-ppm resonance appears as a singlet in the proton undecoupled spectrum.

The methylene resonances show sharp singlets at 36.69 and 32.96 ppm in the proton decoupled spectrum. They appear as a triplet of 1:2:1 intensity in the proton undecoupled spectrum, each multiplet exhibiting very similar additional splittings due to 2-bond couplings to the adjacent methylene protons. Therefore, the assignment was made by comparison with the ^{13}C NMR spectrum of α-ketoglutaric acid deuterated at the 3 position by the method of Cooper & Redfield (1975). The proton decoupled ^{13}C NMR spectrum of this sample included a pentet of peaks of equal intensity centered at 36.40 ppm (average J_{C-D} = 19.6 Hz) and a singlet at 31.54 ppm in the methylene region. Consequently, the low-field resonance (36.69 ppm) was assigned to the C-3 carbon and the high-field resonance (32.96 ppm) was assigned to the C-4 carbon.

As the pH of the α-ketoglutarate solution is lowered below 3, a set of weak resonances increase in intensity. Their chemical shifts have a pH dependence that is slightly different from the resonances of the keto form. These peaks were assigned to the *gem*-diol form by analogy with the proton NMR spectra of other α-keto acids (Cooper & Redfield, 1975). Figure 1c shows the ^{13}C NMR spectrum of α-ketoglutaric acid at pH 0.11. The absorption at 94.80 ppm is characteristic of a *gem*-diol carbon, and it is observed in the ^{13}C NMR spectrum of pyruvic acid and α-ketovaleric acid at this pH. Our studies indicate that both of these compounds exist as an equilibrium mixture of keto and *gem*-diol forms at this pH. The remaining peaks were assigned to the carboxyl carbons and the methylene carbons of the *gem*-diol form of α-ketoglutaric acid by using techniques similar to the one used for the keto form and by following the NMR spectrum closely as a function of pH. The C-3 methylene of the *gem*-diol form is unresolved from the same resonance of the keto form below pH 1.5 and has an intensity equal to the sum of the intensities of the C-4 methylene peaks of the two forms.

The UV absorption spectrum of α-ketoglutaric acid in neutral aqueous solution consists of a weak absorption band centered at 320 nm that is typical of the $^1\Gamma_{n\pi^*}$–$^1\Gamma_1$ electronic transition observed in other α-keto acids (Annett et al., 1973).

(B) Equilibrium Studies. (1) pH Dependence of the Equilibria. The pH dependence of the ^{13}C NMR spectrum of α-ketoglutarate over the pH region 0–7 reveals that the amount of *gem*-diol form in solution depends on the pH and parallels the titration of the two carboxyl groups. From the sharpness of all the carbon resonances (except[2] the C-2 of the keto form below pH 6.0 and the C-1 of the keto form below pH 2.0) of both the keto and *gem*-diol forms during the entire pH range studied, it is evident that their interconversion (k < 0.1 s^{-1}) is *very slow* in the ^{13}C NMR time scale for all the carbons. We have calculated the amount of the *gem*-diol form from the integrated intensities of the spectral peaks, and its dependence on the pH of the solution is shown in Figure 2. We do not observe a separate set of resonances for the cyclic form; the available evidence suggest that the cyclic ⇌ keto interconversion is *extremely rapid* and the resonances assigned to the keto form are actually weighted averages of the peaks

[2] The broadening of the C-2 and C-1 resonances of the keto form will be discussed in detail later in this paper.

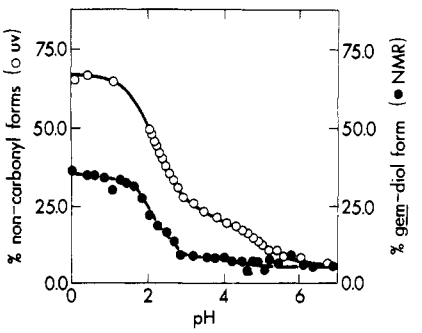

FIGURE 2: Variation in the amount of the noncarbonyl (cyclic and *gem*-diol) forms of α-ketoglutaric acid as a function of pH at 29 °C. (●) ^{13}C NMR data; spectra were obtained in a 1 M solution of α-ketoglutaric acid and the pH was adjusted with a concentrated solution of NaOH. The percent *gem*-diol form is based on the intensities of the hydrate and keto peaks of four resonances, C-1, C-3, C-4, and C-5, and represents an average. The keto peaks are actually weighted averages of the peaks due to keto and cyclic forms. (○) UV absorbance at 320 nm of 25 mM α-ketoglutarate. The keto form absorbs at 320 nm and the cyclic and *gem*-diol forms do not.

arising from the keto and cyclic forms. The cyclic ⇌ *gem*-diol interconversion is very slow for all carbons in the NMR time scale.

The absorbance at 320 nm by α-keto acids also provides a convenient way to measure the amount of *gem*-diol form in solution (Bell, 1966; Ono et al., 1961). Since the carbonyl group is responsible for absorbance at 320 nm, we assumed that the absorbance by the cyclic and *gem*-diol species at this wavelength is negligible and calculated the amount of free form with ϵ_{320} = 29.1 M^{-1} cm^{-1} for the keto form of α-ketoglutaric acid. The molar absorption coefficient was calculated from the absorbance of 25 mM α-ketoglutaric acid at neutral pH by assuming that 6.7% of α-ketoglutaric acid is present as hydrate and cyclic species, a value determined by NMR measurements. Figure 2 shows the percent of species that contain no carbonyl group (*gem*-diol and cyclic forms) as a function of pH.

There are significant differences between the UV and NMR titration curves shown in Figure 2. The UV absorbance titration curve shows that as the pH is lowered from 6.8 to 2.9 the percent of noncarbonyl forms increases from 7% to 29%, while the NMR results show that the percent of the *gem*-diol increases from 7% to 9%. Lowering the pH to 0 to completely protonate the two carboxylate groups of α-ketoglutaric acid leads to an equilibrium mixture of 65% noncarbonyl forms according to UV measurements and only 35% *gem*-diol form according to NMR measurements. The difference between the two numbers is not due to the difference in the total concentration of α-ketoglutarate used for the UV absorbance (25 mM) and ^{13}C NMR (1 M) measurements since the amount of *gem*-diol as measured by ^{13}C NMR (30 ± 2%) is independent of the total α-ketoglutarate concentration in the range 0.025–1 M at a constant pH (0.33 ± 0.07) and temperature (37 ± 1 °C). Since the protonation of the γ-carboxylate group with a pK_a of 4.8 (Jen & Knoche, 1969) leads to very little change in the amount of *gem*-diol as evidenced by the ^{13}C NMR and proton NMR (Cooper & Redfield, 1975) spectra, the large decrease in the carbonyl absorption accompanying the protonation should be due to an increase in the amount of the cyclic form. On this basis, we calculate that α-ketoglutaric acid in the fully protonated state exists as an equilibrium mixture of 35% keto, 35% *gem*-diol, and 30% cyclic forms in aqueous solution at 29 °C.

Although our results on the amount of keto form are in very good agreement with the values reported by Cooper & Red-

Table I: Thermodynamic Parameters, Rate Constants at 0 °C, and Activation Energies[a] for the Hydration[b] of α-Ketoglutarate in Methanol-Water Mixed Solvents

exptl conditions[c]	[KG] (M)	$\Delta H^{\circ\prime}$ (kcal mol^{-1})	$\Delta S^{\circ\prime}$ (eu)	k_1 (min^{-1}) [E_a (kcal)]	k_{-1} (min^{-1}) [E_a (kcal)]	$k = k_1 + k_{-1}$ (min^{-1}) [E_a (kcal)]
1	0.1	−6.2 ± 0.2	−25.9 ± 1.8	0.038 [7.0]	0.18 [13.1]	0.22 [11.2]
2	0.1	−4.5 ± 0.3	−12.8 ± 2.2	0.99 [7.2]	0.14 [11.6]	1.13 (7.4 kcal)
1	0.01	−5.8 ± 0.3	−24.4 ± 2.2	0.037 [7.0]	0.18 [13.4]	0.22 (11.3 kcal)

[a] The rate constants and the activation energies are subject to 5% error. [b] The lactol form was neglected in calculating all the parameters described above. [c] Experimental condition 1: α-ketoglutarate in 0.01 M potassium phosphate buffer [pH 7.6 (the pH quoted is the pH of the aqueous buffer at 25 °C before the addition of methanol)] containing 40% v/v methanol. Experimental condition 2: α-ketoglutaric acid in 0.1 M HCl solution containing 60% v/v methanol.

field (1975) for α-ketoglutaric acid under similar conditions, their value for the amount of *gem*-diol (53%) form differs significantly from ours. Our studies also lead to lower values for the amount of *gem*-diol in pyruvic acid (41 ± 4% vs. 65%) and α-ketovaleric acid (28 ± 4% vs. 53% for α-ketoisovaleric acid) compared to the values reported by Cooper & Redfield (1975). The equilibria are highly temperature dependent and a part of the difference may be due to the 5 °C difference in the temperature between our measurements and theirs. It is interesting to note from our results that while the γ-carboxylate protonation leads to a large increase in the amount of the cyclic form, the protonation of α-carboxylate group with a pK of 1.8 (Jen & Knoche, 1969) leads to a large increase in the amount of the *gem*-diol form and a smaller increase in the amount of the cyclic form. The amount of *gem*-diol becomes pH independent below pH 1 and above pH 6 and remains at about 35% and 7%, respectively.

(*2*) *Temperature Dependence of the Equilibria.* The temperature dependence of the equilibrium between the keto and the *gem*-diol forms of α-ketoglutaric acid was also studied by monitoring its UV absorbance in an aqueous methanol solution at neutral and low pH. The presence of methanol does not alter the equilibrium significantly. The thermodynamic parameters obtained are shown in Table I. Since the amount of cyclic form is negligible at the neutral pH, we treated the data as for a simple keto ⇌ *gem*-diol equilibrium. The equilibrium constant and the ΔH° for the reaction appear to be concentration independent at the neutral pH.

(*C*) *Kinetics of Keto ⇌ gem-Diol Interconversion in α-Ketoglutarate.* The temperature dependences of the rate constants k_1 and k_{-1} (see Scheme I) were measured under various conditions, and the activation energies calculated from the Arrhenius plots (which were linear in the temperature region −50 to 30 °C) are shown in Table I. The rate constants are independent of the total α-ketoglutarate concentration, proving that the absorbance changes are not due to the dimerization reactions mentioned by Kumashiro (1960). The effects of buffer components on the rate constants for the hydration were also studied since buffer catalysis of addition rections to the carbonyl group (Jencks, 1969) is well-known. These are shown in Figure 3 for phosphate and Tris buffers. It appears that Tris buffer is more effective as a general acid–base catalyst than phosphate.

(*D*) *Kinetics of the Cyclization Reaction of α-Ketoglutaric Acid.* The pH dependence of the ^{13}C NMR spectrum of α-ketoglutarate (Figure 1a,b) indicated that the C-2 resonance of the *keto* form broadens below pH 6.0. The broadening is specific for this resonance at this pH, and it increases exponentially with decreasing pH until at pH below 4.0 it completely broadens out. A similar phenomenon was observed for

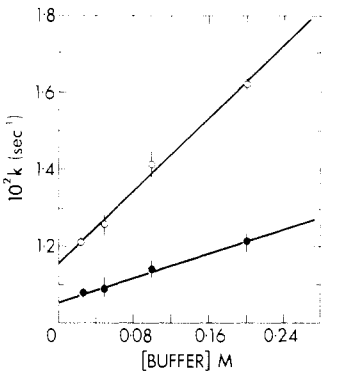

FIGURE 3: Catalysis of the hydration reaction of α-ketoglutaric acid (0.01 M) by buffer components phosphate (●) and Tris (○) at 3.5 °C and at pH 7.6.

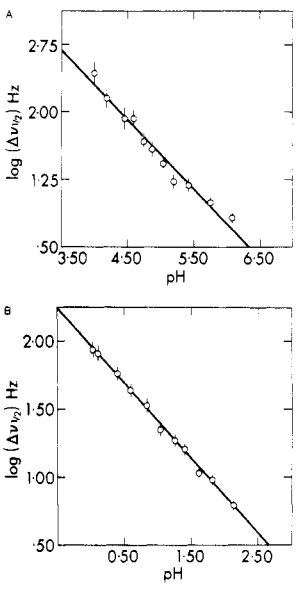

FIGURE 4: Line width of the C-2 (A) and C-1 (B) resonances of the keto form of α-ketoglutarate as a function of pH. The line broadening is due to the decrease in the pseudo-first-order rate constant for the keto ⇌ cyclic interconversion as the pH is lowered and represents a transition from the "very fast exchange" to "moderately fast exchange" region.

the C-1 resonance of the keto form below pH 2.0. The pH dependence of the line widths is shown in Figure 4. The line widths remained constant at 5–6 Hz for C-2 (keto) above pH 6.5, for C-1 (keto) above pH 2.5, and for all other resonances at all pH values between 0 and 10. We have assigned the source of the broadening to the exchange between the cyclic and the keto forms for the following reasons.

(1) The C-2 and C-1 resonances of the *gem*-diol form appear

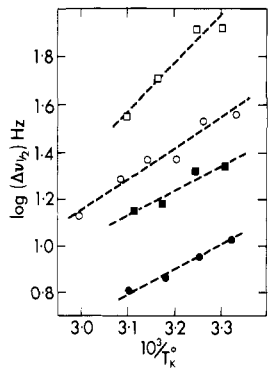

FIGURE 5: Temperature dependence of the ^{13}C NMR line widths of α-ketoglutarate (1 M) at 50.32 MHz. (○) C-1 (keto) resonance at pH 1.02; (□) C-2 (keto) resonance at pH 4.53; (■) C-2 (keto) resonance at pH 5.14; (●) C-2 (keto) resonance at pH 5.66. The data were collected with 16K data points and a spectral width of 12 kHz.

as sharp peaks whose line widths are pH independent in the pH range 0–10. The first-order rate constant for the hydration of α-ketoglutarate measured spectrophotometrically at pH 4 is only 20% higher than the value at pH 6. Thus the large pH-dependent broadening observed for the C-2 and C-1 resonances of the keto form cannot be due to the keto $\rightleftharpoons$ *gem*-diol interconversion.

(2) The C-2 and C-1 resonances of both the keto and *gem*-diol forms of α-ketovaleric acid are sharp at acidic and at neutral pH. This compound cannot form a cyclic species but it can form an enol. The absence of line broadening in this compound indicates that the acid-catalyzed enolization is not the source of the C-2 line broadening for the keto form of α-ketoglutarate.

(3) Cooper & Redfield (1975) noticed an asymmetry and considerable distortion in the proton NMR signals of the β-methylene protons of α-ketoglutaric acid at pH 0.5. The distortions were restricted to the keto form, and they concluded that it was due to the moderately rapid interconversion between cyclic and acylic structures.

(4) The temperature dependence of the C-2 resonance line widths was studied at pHs 5.66, 5.14, and 4.53 in the temperature region 27–50 °C. These are shown in Figure 5. The line widths decrease with increasing temperature at all these pHs, indicating "very fast exchange" above pH 6.5 for this resonance (Reeves, 1965). A semilogarithmic plot of the line widths against $1/T$ (Figure 5) is linear in the temperature region studied. The temperature dependence of the C-1 resonance line width studied at pH 1.02 also exhibits a similar behavior (Figure 5), indicating very fast exchange for this resonance above pH 2.5. Since the *gem*-diol $\rightleftharpoons$ keto interconversion is too slow to have any effect on the NMR spectra, this extremely rapid exchange should be an intramolecular process such as cyclization.

(5) The increase in the line width of the C-2 (keto) resonance of α-ketoglutarate is accompanied by large upfield shifts of this resonance. Its chemical shift changes from 206.74 ppm at pH 6.36 to 199.23 ppm at pH 4.20 to a net upfield shift of 7.51 ppm. In this pH region the γ-carboxylate of α-ketoglutarate (pK = 4.8) is expected to be protonated and its protonation should lead to a C-2 resonance upfield shift of only 1.95 ppm as observed in a model compound, γ-ketovaleric acid, which apparently does not cyclize. Therefore, the large shift of the C-2 (keto) resonance must be due to some other process. Since the C-2 resonance of the cyclic form is expected to have a chemical shift similar to the chemical shift of this resonance in the *gem*-diol form (96 ppm), the large upfield shifts of the C-2 (keto) resonance must be due to the increase in the amount of the cyclic form at low pH. Another line of evidence leading to the same conclusion is based on the comparison of the C-2 chemical shifts in α-ketoglutaric acid and α-ketovaleric acid. The C-2 chemical shifts for these two compounds in the fully ionized form at neutral pH are 206.74 and 208.93 ppm. Protonation of the α-carboxylate shifts this resonance 9.53 ppm upfield to a chemical shift of 199.40 ppm in α-ketovaleric acid. The C-2 chemical shift in α-ketoglutaric acid however, is 199.23 ppm at pH 4.2, nearly 2.5 pH units above the pK for the protonation of the α-carboxylate. Thus, the lower values for the chemical shift of C-2 (keto) resonance in α-ketoglutaric acid indicates the presence of significant amounts of the cyclic form.

Figure 4 shows that lowering the pH leads to an increase in the line widths of the C-2 and C-1 resonances at different pH regions. Since the temperature dependence of these line widths reveals that the keto $\rightleftharpoons$ cyclic interconversion is in the very fast exchange region for all the resonances above pH 6.5, the increase in the line width of the C-2 resonance below pH 6.0 and that of the C-1 resonance below pH 2.0 must be due to a decrease in the first-order rate constant for this interconversion. The decrease in the first-order rate constant for the exchange process with decreasing pH suggests the presence of a hydroxide ion concentration term in the first-order rate constant.

The difference in the pH at which C-2 and C-1 resonances begin to broaden is related to the difference in the chemical shifts ($\nu_{keto} - \nu_{cyclic}$) of these resonances in the keto and cyclic forms. An approximate magnitude of this quantity for the C-2 resonance is 5500 Hz at the resonance frequency of 50.32 MHz. It is about 500 Hz for C-1 resonance and less than 50 Hz for C-3, C-4, and C-5. The critical rate constant for exact collapse of the resonances of the keto and cyclic forms to a single resonance (Reeves, 1965) is

$$k' = 2^{1/2}\pi(\nu_{keto} - \nu_{cyclic}) \quad (3)$$

which is approximately 2.4×10^4 s^{-1} for the C-2 resonance and 1 order of magnitude less for C-1 and 2 orders of magnitude less for other resonances. The above calculation is, however, strictly valid only if the amount of keto and cyclic forms are equal. The pH and temperature dependence of the equilibrium and the lack of knowledge about the exact amount of the two forms and the individual chemical shifts of the resonances in the two forms precludes detailed analysis of the data to yield more accurate rate constants.

(II) Utilization of α-Ketoglutaric Acid Forms for Reductive Amination by GDH. (A) Does GDH Catalyze the Hydration Reaction of α-Ketoglutaric Acid? We measured the first-order rate constant for the hydration of α-ketoglutarate (2.5 mM) in 0.1 M potassium phosphate buffer (pH 7.6) at 3.2 °C and at several enzyme concentrations (0–93 μg/mL) with and without NADPH (1.13×10^{-4} M). Within the limits of error (5%), the rate constants were all equal and the average was 0.63 ± 0.03 min^{-1}. α-Ketoglutarate binds only weakly to GDH to form a binary complex, but its binding to GDH–NADPH to form a ternary complex is quite strong (Cross, 1972). Thus, the results show that the enzyme does not accelerate the conversion of the *gem*-diol form to the keto form.

(B) Does GDH Show Specificity for the Keto Form of α-Ketoglutarate? Figure 6 shows the rate of decrease of the coenzyme absorption at 340 nm when the reductive amination reaction catalyzed by GDH is initiated by (a) α-ketoglutarate predominantly in the keto form and (b) α-ketoglutaric acid predominantly in the noncarbonyl (cyclic and *gem*-diol) forms. The concentration of α-ketoglutaric acid is rate limiting in

Table II: Amount of NADPH Oxidized in the First Three Minutes of the GDH-Catalyzed Reductive Amination of α-Ketoglutarate for Initiation by α-Ketoglutarate[a] Solutions of Various Initial Species Distribution[b]

initial ketoglutarate solution	initial species distribution (%)			differences in amount of NADPH oxidized		
	keto	*gem*-diol	lactol or cyclic form	solutions compared	ΔA_{340} (0–3 min)	jumps involved
1	93 ± 3	6 ± 3	1 ± 3	1 and 1	0 (control)	none
2	93 ± 3	6 ± 3	1 ± 3	2 and 2	0 (control)	none
3	32 ± 3	combined 68 ± 3		3 and 2	0.0086 ± 0.0014	temp
				3 and 1	0.0102 ± 0.0008	temp
4	35 ± 3	35 ± 3	30 ± 3	4 and 1	0.0060 ± 0.0006	pH
5	35 ± 3	35 ± 3	30 ± 3	5 and 2	0.0054 ± 0.0006	pH
6	2.5 ± 3	combined 97.5 ± 3		6 and 1	0.0130 ± 0.0010	pH, temp
				6 and 2	0.0118 ± 0.0006	pH, temp

[a] See the text for details. [b] The final species distribution is as shown for solutions 1 and 2. The amount of methanol in the final solution is 0.6% v/v for experiments with solutions 2, 3, 5, and 6. There is no methanol in the final solution for experiments with solutions 1 and 4. Other experimental conditions are as described in the legend for Figure 6.

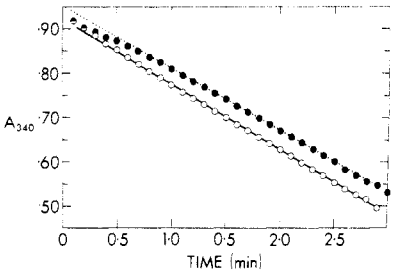

FIGURE 6: Depletion of NADPH when the GDH-catalyzed reductive amination of α-ketoglutarate was initiated with (○) α-ketoglutarate in the keto form and (●) α-ketoglutarate in the nonketo forms; [NADPH] = 1.52×10^{-4} M; [NH_4Cl] = 0.050 M; [α-ketoglutaric acid] = 0.15 mM; [GDH] = 1.6 μg/mL. The reaction was carried out at 15.1 °C in a 0.1 M potassium phosphate buffer of pH 7.6. The keto form of α-ketoglutarate was generated by incubating α-ketoglutarate in pH 7.6 buffer for 1 h at 15 °C. The nonketo form of α-ketoglutarate (contains only 2.5% keto form) was generated by incubating a solution of α-ketoglutarate in 0.1 M HCl and 60% v/v methanol at −49.6 °C in a Neslab cryogenic bath for 22 h.

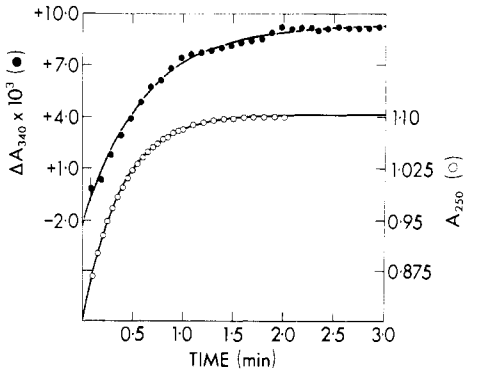

FIGURE 7: Difference between the NADPH absorption at 340 nm for the two modes of initiation described in the legend for Figure 6 and UV absorption (250 nm) of α-ketoglutaric acid (0.01 M) in potassium phosphate buffer (pH 7.6) at 15.1 °C as a function of time. The change in the absorbance of α-ketoglutaric acid represents the conversion of the *gem*-diol form to the keto form after a pH jump from pH 1.3 to 7.6.

these experiments. The figure shows that the enzymatic reaction is much slower when it is initiated by noncarbonyl forms of α-ketoglutaric acid relative to the rate observed when the reaction is initiated by the keto form with the total α-ketoglutarate concentration being equal in both cases. The rates in the two cases become equal within a few minutes after initiation. This indicates the conversion of a form that was not a substrate at time zero to a form that is a substrate, over a period of a few minutes. The approach of the difference in the rates for the two modes of initiation to zero or a constant value (if the temperature and the concentrations of enzyme and α-ketoglutaric acid are not *identical* in both runs) indicates the time scale for the conversion of the nonsubstrate form to the substrate form. Alternatively, the difference in the coenzyme absorbance at 340 nm between the two modes of initiation at the same total α-ketoglutarate concentration as a function of time provides the same kinetic information. This is shown in Figure 7 along with the first-order approach to equilibrium of α-ketoglutarate species after a pH perturbation at the same temperature. The first-order rate constant for the processes are 2.7 ± 0.2 min^{-1} and 1.8 ± 0.7 min^{-1} in the nonenzymatic and enzymatic systems, respectively. The nonenzymatic conversion of the lactol to the keto form is quite rapid, and it is complete within the mixing time (5 s). Consequently, the kinetic process shown in Figure 7 represents the conversion of the *gem*-diol form to the keto form. The good agreement of the rate constants in the enzymatic and nonenzymatic systems indicates that the keto form is the substrate and that the *gem*-diol form has no significant substrate activity under these conditions.

The conversion of *gem*-diol to the keto form should be virtually complete in about 3 min at 15 °C. Consequently, the amount of NADPH oxidized in the first 3 min of the enzymatic reaction at a fixed rate limiting concentration of α-ketoglutarate should differ for different initial speecies composition of α-ketoglutarate. So that this hypothesis could be tested, the enzyme-catalyzed reaction was initiated by using a fixed amount of α-ketoglutaric acid in (1) 0.1 M phosphate buffer (pH 7.6) containing no methanol at 15 °C, (2) 0.1 M phosphate buffer (pH 7.6) containing 60% v/v methanol at 15 °C, (3) solution 2 at −50 °C, (4) 0.10 M HCl solution containing no methanol at 15 °C, (5) 0.1 M HCl solution containing 60% v/v methanol at 15 °C, and (6) solution 5 at −50 °C. Experiments 1 and 2 are control experiments that involve virtually no change in pH[3] and temperature. Experiment 3 involves a temperature jump of about 65 °C, and experiments 4 and 5 involve a pH jump of about 6 units. Experiment 6 involves a combined pH and temperature jump. The difference between the amount of NADPH oxidized in the first 3 min of the reaction between experiments 3–6 and an appropriate control experiment 1 or 2 is shown in Table II.

Table II shows that the combined pH and temperature-jump experiment leads to the largest difference in the extent of the reaction as expected for a change in the keto form from 2.5%

[3] There is a slight change in the pH of α-ketoglutarate in a buffered solution when the amount of methanol is changed from 60% v/v to 0.6% v/v. This change is quite small, however, and leads to very small changes in the species distribution at neutral pH.

and 93%. However, the individual pH jump and temperature-jump experiments yield quite different numbers although in both experiments the amount of keto form (35% and 32%, respectively) at the start is nearly the same. This is not surprising since it is the amount of *gem*-diol form (the cyclic form converts to keto form very rapidly) at the start which determines the differences in the amount of NADPH oxidation in the first 3 min of the reaction, and it is different for the two cases. The low pH solutions (cases 4 and 5) contain much less *gem*-diol than the low-temperature solution (case 3) because there is a greater amount of cyclic form (30%) in the low-pH solution than in the low-temperature solution. Thus, there is a qualitative inverse correlation between the extent of the reaction in the first 3 min and the amount of *gem*-diol in the α-ketoglutarate solution used to initiate the enzyme reaction.

The results described above show clearly that the keto form of α-ketoglutarate is the substrate for GDH, that the *gem*-diol form is not a substrate, that it becomes a substrate only after nonenzymatic conversion to the keto form, and that this nonenzymatic conversion is accelerated neither by the enzyme nor by the enzyme–NADPH complex with or without ammonia present in solution. The *gem*-diol form meets the structural requirements for an inhibitor competitive with L-glutamate (Caughey et al., 1957). Indeed, it has been shown (Tienhaara & Meany, 1973) that the *gem*-diol form of pyruvic acid is an inhibitor of lactate dehydrogenase. We do not know yet whether the *gem*-diol form of α-ketoglutarate is actually an inhibitor of the GDH reaction. Although we know that α-ketoglutarate causes substrate inhibition at high concentrations (Bates & Frieden, 1973), and abortive enzyme–NADP–α-ketoglutarate complexes are known to be inhibitory (Colen, 1978), the specific species of α-ketoglutarate involved in forming these complexes are still unknown.

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