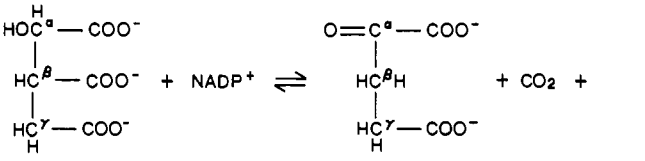
Kaplan, N. O., Ciotti, M. M., Stolzenbach, F. E., & Bachur, N. R. (1955) *J. Am. Chem. Soc. 77*, 815–816.
Kaplan, N. O., Ciotti, M. M., & Stolzenbach, F. E. (1956) *J. Biol. Chem. 221*, 833–844.
Michelson, A. M. (1964) *Biochim. Biophys. Acta 91*, 1–13.
Nambiar, K. P., Stauffer, D. M., Kolodkiej, P. A., & Benner, S. A. (1983) *J. Am. Chem. Soc. 105*, 5886–5890.
Oppenheimer, N. J. (1982) in *Pyridine Nucleotide Coenzymes* (Everse, J., Anderson, B., & You, K.-S., Eds.) pp 51–89, Academic, New York.
Oppenheimer, N. J. (1984) *J. Am. Chem. Soc. 106*, 3032–3033.
Oppenheimer, N. J. (1986a) in *Mechanisms of Enzymatic Reactions: Stereochemistry* (Frey, P. A., Ed.) pp 15–28, Elsevier, New York.
Oppenheimer, N. J. (1986b) *J. Biol. Chem. 261*, 12209–12212.
Oppenheimer, N. J., & Kaplan, N. O. (1975) *Arch. Biochem. Biophys. 166*, 526–535.
Oppenheimer, N. J., & Kaplan, N. O. (1976) *Biochemistry 15*, 3981–3989.
Oppenheimer, N. J., Arnold, L. J., & Kaplan, N. O. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 3200–3205.
Oppenheimer, N. J., Arnold, L. J., Jr., & Kaplan, N. O. (1978) *Biochemistry 17*, 2613–2619.
Orr, G. A., & Blanchard, J. S. (1984) *Anal. Biochem. 142*, 232–234.
Rodkey, F. L. (1955) *J. Biol. Chem. 213*, 777–786.
Saneyoshi, M. (1971) *Chem. Pharm. Bull. 19*, 493–498.
Sarma, R. H., & Mynott, R. J. (1972) *Org. Magn. Reson. 4*, 577–584.
Walt, D. R., Findeis, M. A., Rios-Mercadillo, V. M., Auge, J., & Whitesides, G. M. (1984) *J. Am. Chem. Soc. 106*, 234–239.
Woenckhaus, C., & Jeck, R. (1970) *Justus Liebigs Ann. Chem. 736*, 126–133.
Woenckhaus, C., & Zumpe, P. (1965) *Biochem. Z. 343*, 326–328.
Woenckhaus, C., Volz, M., & Pfleiderer, G. (1964) *Z. Naturforsch., B: Anorg. Chem., Org. Chem., Biochem., Biophys., Biol. 19B*, 467–470.
Yoshikawa, M., Kato, T., & Takenishi, T. (1967) *Tetrahedron Lett.*, 5065–5068.
You, K.-S., Arnold, L. J., Jr., Allison, W. S., & Kaplan, N. O. (1978) *Trends Biochem. Sci. (Pers. Ed.) 3*, 265–268.

Ionization of Isocitrate Bound to Pig Heart $NADP^+$-Dependent Isocitrate Dehydrogenase: ^{13}C NMR Study of Substrate Binding†

Robert S. Ehrlich and Roberta F. Colman*
Department of Chemistry, University of Delaware, Newark, Delaware 19716
Received November 6, 1986; Revised Manuscript Received January 20, 1987

ABSTRACT: Isocitrate and α-ketoglutarate have been synthesized with carbon-13 enrichment at specific positions. The ^{13}C NMR spectra of these derivatives were measured as a function of pH. The magnitudes of the changes in chemical shifts with pH for free isocitrate and the magnesium–isocitrate complex suggest that the primary site of ionization is at the β-carboxyl. In the presence of the enzyme $NADP^+$-dependent isocitrate dehydrogenase and the activating metal magnesium, the carbon-13 resonances of all three carboxyls remain constant from pH 5.5 to pH 7.5. Thus, the carboxyls remain in the ionized form in the enzyme–isocitrate complex. The α-hydroxyl carbon resonance could not be located in the enzyme–isocitrate complex, suggesting immobilization of this group. Magnesium produces a 2 ppm downfield shift of the β-carboxyl but does not change the resonances of the α- and γ-carboxyls. This result is consistent with metal activation of both the dehydrogenation and decarboxylation reactions. The ^{13}C NMR spectrum of α-ketoglutarate remains unchanged in the presence of isocitrate dehydrogenase, implying the absence of alterations in geometry in the enzyme-bound form. Formation of the quaternary complex with Mg^{2+} and NADPH leads to loss of the α-ketoglutarate resonances and the appearance of new resonances characteristic of α-hydroxyglutarate. In addition, a broad peak ascribed to the enol form of α-ketoglutarate is observed. The substantial change in the shift of the β-carboxyl of isocitrate and the lack of significant shifts in the other carboxyls of isocitrate or α-ketoglutarate suggest that interaction of the β-carboxyl with the enzyme contributes to the tighter binding of isocitrate and may be significant for the oxidative decarboxylation function of isocitrate dehydrogenase.

The oxidative decarboxylation of isocitrate to form α-ketoglutarate by $NADP^+$-dependent isocitrate dehydrogenase from pig heart [*threo*-D_s-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] requires a divalent metal (Villafranca & Colman, 1972; Colman, 1983):



†Supported by U.S. Public Health Service Grant DK-39075 and grants-in-aid from the American Heart Association, Southeastern Pennsylvania Chapter, and the American Heart Association of Delaware, Inc.

0006-2960/87/0426-3461$01.50/0

Either isocitrate or metal can bind to the enzyme in the absence of the other ligand, but binding of each ligand is tightened at least fivefold in the ternary complex (Villafranca & Colman, 1972; Colman, 1969). Measurements of dissociation constants as a function of pH suggest that isocitrate binds in a form in which all three carboxyl groups are ionized (Ehrlich & Colman, 1976; Colman, 1972a).

In this study ^{13}C NMR has been used as a probe of the ionization of the carboxyl groups of isocitrate when it is free and enzyme bound. The resonance positions of carboxyl carbons are sensitive to the state of ionization (Hagen & Roberts, 1969). Perturbation of the resonances of enzyme-bound isocitrate by magnesium was investigated to examine the factors leading to tighter binding of isocitrate in the presence of metal. Because of the low sensitivity of ^{13}C NMR, specific carbons of the ligand were isotopically enriched in ^{13}C, according to the notation of eq 1.

Direct binding studies have been made of α-ketoglutarate binding to isocitrate dehydrogenase (Colman, 1969). The ^{13}C NMR spectrum of α-ketoglutarate has been measured in an attempt to see alterations in the carboxyls or carbonyl resonances of this ligand. Isotope-exchange studies (Rose, 1960) indicate that a keto–enol transition of α-ketoglutarate is promoted by the enzyme in the presence of metal and reduced nucleotide. Evidence for this transformation has now been found from ^{13}C NMR. A preliminary report on some of this work has been presented (Ehrlich & Colman, 1986).

Experimental Procedures

Materials. Isocitrate dehydrogenase was prepared as previously described (Bacon et al., 1981). The enzyme was concentrated to 15–30 mg/mL either by using Amicon ultrafiltration concentrators with PM-10 membranes or with Centricon concentration devices (Amicon). The enzyme was dialyzed against the indicated buffers containing 10% glycerol and 10% D_2O. An extinction coefficient of 1.08 mg^{-1} at 280 nm (Johanson & Colman, 1981) and a molecular weight of 58 000 (Colman, 1972b) were used to determine enzyme concentration.

Glutamate dehydrogenase (beef liver), lactate dehydrogenase (hog muscle), glucose 6-phosphate dehydrogenase (yeast) and 6-phosphogluconate dehydrogenase (yeast) were obtained from Boehringer-Mannheim. Unenriched isocitrate and α-ketoglutarate were from Sigma, as were pyruvate, NADase (*Neurospora crassa*), and all nucleotides. DL-Glutamate enriched with ^{13}C (91%) in the α-carbon was obtained from Prochem. DL-Glutamate enriched in the α-carboxyl (99%) and glutamate enriched in the γ-carboxyl (99%) were obtained from Stohler/Kor Stable Isotopes. $NaH^{13}CO_3$ and $Ba^{13}CO_3$ were from Cambridge Isotope Laboratories.

Preparation of α-Ketoglutarate. ^{13}C-Enriched forms of glutamate were converted to α-ketoglutarate by using glutamate dehydrogenase with NAD^+ as the coenzyme. DL-Glutamate (500 μmol) was dissolved in 100 mL of 0.01 M sodium phosphate (pH 8.0). NAD^+ (2 mM) and glutamate dehydrogenase (2 mg) were added. Lactate dehydrogenase (1 mg) was included to regenerate NAD^+ upon addition of pyruvate. The absorbance at 340 nm was followed, and pyruvate was added in 20-μmol aliquots. When no further increases in optical density at 340 nm were observed, the mixture was loaded on a 1.5 × 8 cm DE-52 column (Whatman) equilibrated with triethylamine–0.01 M acetate (pH 5.5). A linear gradient from 75 mL of equilibration buffer to 75 mL of triethylamine–0.5 M acetate (pH. 5.5) was applied. Fractions were tested for α-ketoglutarate by using glutamate dehydrogenase and an assay mixture containing 0.1 mM NADH, 0.05 M NH_4Cl and 0.1 mM EDTA[1] in triethanolamine–0.05 M acetate (pH 8.0). The presence of nucleotides was determined spectroscopically. The purity of α-ketoglutarate was assessed by ^{13}C NMR. Purification attempts using bicarbonate buffer resulted in multiple products upon lyophilization with substantial decarboxylation of the product, but lyophilization of solutions of α-ketoglutarate in triethylammonium acetate proved satisfactory. The yield of α-ketoglutarate was 20% in terms of L-glutamate.

Conversion of α-Ketoglutarate to Isocitrate. Although the equilibrium of the isocitrate dehydrogenase reaction lies far in the direction of α-ketoglutarate, as much as a 15% yield of isocitrate was obtained by means of the enzyme-catalyzed reaction. α-Ketoglutarate (2 mM) was dissolved in 5 mM sodium phosphate buffer containing 5 mM magnesium sulfate, 0.1 M sodium bicarbonate, 0.5 mM glucose 6-phosphate and 0.5 mM 6-phosphogluconate. The mixture was placed in a plastic bottle and quickly titrated to approximately pH 6.5–7.0. NADPH (1 mM) and 100 units (Bacon et al., 1981) of isocitrate dehydrogenase were added, as well as 0.3 mg of glucose 6-phosphate dehydrogenase and 0.3 mg of 6-phosphogluconate dehydrogenase in order to regenerate the NADP. Carbon dioxide was produced by adding dry ice to the incubation. The bottle was sealed to maintain a pressure of slightly over 1 atm of CO_2. After 4 h, NADase (*Neurospora*) was added to destroy residual $NADP^+$ (which partially overlaps isocitrate in the subsequent column chromatography). For isocitrate labeled in the β-carboxyl, unenriched α-ketoglutarate (5 mM) and 0.1 M $NaH^{13}CO_3$ were used, and other components were identical. The mixture was pressurized by adding HCl to $Ba^{13}CO_3$ contained in a small tube within the reaction bottle.

Isocitrate was purified by chromatography on a 1.5 × 8 cm DE-52 column equilibrated with 0.01 M ammonium bicarbonate. The sample was diluted fivefold before being applied to the column and eluted with a linear gradient from the equilibration buffer (100 mL) to 0.5 M ammonium bicarbonate (100 mL). Fractions were assayed for α-ketoglutarate as described above and for isocitrate by using isocitrate dehydrogenase in an assay mixture containing 0.1 mM $NADP^+$ and 2 mM manganese sulfate in 0.05 M triethanolamine hydrochloride (pH 7.2). Fractions containing isocitrate were lyophilized before use.

NMR Spectroscopy. ^{13}C spectra were obtained in a Bruker WM 250 spectrophotometer at 62.87 MHz with a 10-mm probe. A sweep width of 16 128 Hz was used with 8064 data points. Longitudinal relaxation times of carboxyl and carbonyl groups were found to be about 40 s. A pulse width of 22° and relaxation delay of 2–3 s were routinely used. For carbons with attached protons, a relaxation delay of 0.5 s was used. The decoupling power was decreased during the delay period to reduce sample heating. The signal-to-noise ratio was enhanced by using an exponential line broadening of 6 Hz. Sample volumes were 1.8–2.5 mL. Samples containing enzyme were not spun to avoid denaturation. EDTA was added to samples to chelate paramagnetic metals that might be present. Above pH 5.5, recovery of enzyme activity was at least 90% following a 12-h experiment.

Results

Natural abundance carbon-13 NMR spectroscopy of isocitrate indicates that the resonances arising from the carboxyl groups overlap at some pH values. While assignment of peaks

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid.

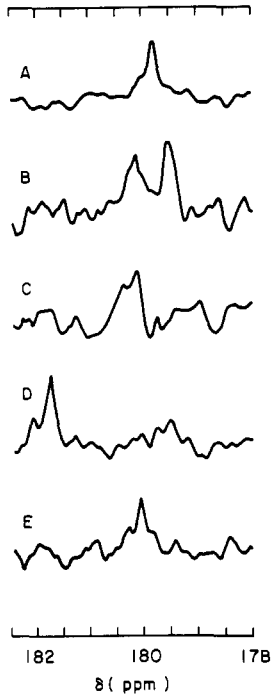


FIGURE 1: ^{13}C NMR spectra of enzyme-bound isocitrate. Samples were in 50 mM MOPS (pH ≥6.7) or 50 mM MES (pH ≤6.7) containing 10% glycerol, 10% D_2O, and 0.1 mM EDTA. Conditions for obtaining spectra are given under Experimental Procedures. The carbon-13 enrichment, pH, and sample contents are as indicated: (A) α-carboxyl, pH 8.0, 0.28 mM isocitrate dehydrogenase and 0.33 mM isocitrate, 24 360 scans; (B) α-carboxyl, pH 6.4, 0.28 mM enzyme and 0.35 mM isocitrate, 24 500 scans; (C) β-carboxy, pH 5.9, 0.42 mM enzyme and 0.47 mM isocitrate, 20 000 scans; (D) β-carboxyl, pH 7.2, 0.42 mM enzyme, 0.47 mM isocitrate, and 5 mM magnesium sulfate, 24 000 scans; (E) γ-carboxyl, pH 6.5, 0.40 mM enzyme and 0.42 mM isocitrate, 13 000 scans.

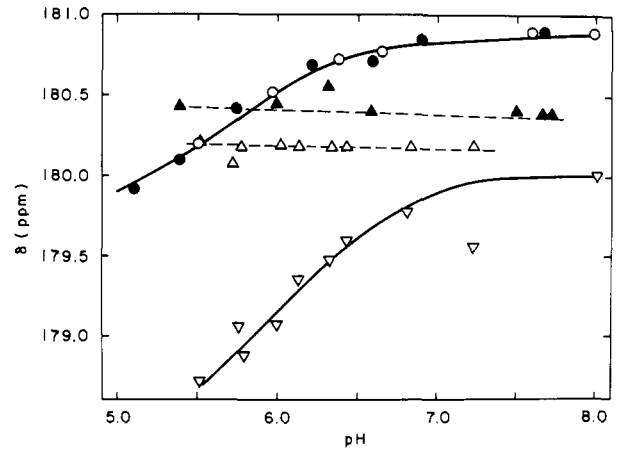


FIGURE 2: pH dependence of the chemical shift of the α-carboxyl of isocitrate. In the absence of enzyme the chemical shifts in the absence of magnesium (○) and in the presence of 5 mM $MgSO_4$ (●) are fit (solid line) to a standard titration curve by using shifts for the protonated and unprotonated forms and pK given in Table I. In the presence of enzyme the shifts with 5 mM $MgSO_4$ (▲) and without magnesium (△, ▽) are shown. The dashed lines (- - -) are drawn arbitrarily. The pH-dependent resonance in the absence of magnesium (▽) is fit (solid line) by using a protonated shift δ_p = 178.2, an unprotonated shift δ_u = 180.0, and a pK of 5.96.

Table I: Titration of Resonances of Isocitrate in the Absence of Enzyme[a]

resonance	δ_p[b]	δ_u[b]	pK
α-carboxyl	179.67 ± 0.14	180.88 ± 0.02	5.64 ± 0.11
β-carboxyl	177.78 ± 0.60	181.38 ± 0.04	5.21 ± 0.13
γ-carboxyl	179.13 ± 0.40	181.50 ± 0.03	5.48 ± 0.13

[a] These values were calculated for the α- and γ-carboxyls from data obtained in the absence and presence of 5 mM $MgSO_4$. For the β-carboxyl, only data obtained in the presence of 5 mM $MgSO_4$ were used. [b] The chemical shifts of the protonated and unprotonated carboxyl groups are δ_p and δ_u, respectively.

could be made by matching their pH dependences with the pH dependences of the resonances of adjacent, nonionizing carbons, it is simpler to make assignments by using the carbon-13 enriched derivatives synthesized for use in the studies with isocitrate dehydrogenase.

Observation of enzyme-bound resonances for carbons of isocitrate requires that line broadening be small. The carbon-13 resonances of free isocitrate have widths of less than 10 Hz and are limited by magnet homogeneity so that the natural widths are lower. In the presence of approximately stoichiometric enzyme concentrations, line widths for all carboxyls remain narrow (10–15 Hz when corrected for the 6-Hz line broadening that was introduced to enhance signal to noise). Representative spectra are shown in Figure 1. In the absence of metal, a single bound resonance was observed (Figure 1A,C,E) except for the α-carboxyl, which splits as the pH is lowered (Figure 1B). In the presence of 5 mM magnesium, a single resonance was observed for all carboxyls in the pH range 5.5–8.0 (e.g., Figure 1D). When isocitrate is added in excess of enzyme, the lines broaden to about 90 Hz at an isocitrate-to-enzyme ratio of 1.6:1. This observation indicates the presence of intermediate rates of exchange between free isocitrate and bound isocitrate. The broadening precludes accurate measurement of shifts, and most experiments were performed with stoichiometric levels of isocitrate and enzyme.

^{13}C NMR Spectroscopy of the α-Carboxyl of Isocitrate. In the absence of isocitrate dehydrogenase, the resonance of the α-carboxyl shifts upfield as the pH is lowered from 8 to 5 (Figure 2). The chemical shift is unaltered by the addition of the diamagnetic metal ion magnesium (● as compared with ○). By use of nonlinear least-squares fit employing the Marquardt algorithm (Marquardt, 1963), the pK and shifts for protonated and unprotonated isocitrate may be calculated, with the results presented in Table I. Separate fits to the data with and without $MgSO_4$ gave values within the standard errors shown in Table I for the least-squares fit to the combined data.

As can be seen from the plot of resonance position against pH for enzyme-bound isocitrate in the presence of magnesium sulfate and isocitrate dehydrogenase, the chemical shift remains constant at δ = 180.4 over the pH range 5.4–7.7 (Figure 2, ▲). It is most likely that the α-carboxyl remains ionized in a single environment on the enzyme.

When magnesium is omitted and fortuitous metal is removed with EDTA, a single upfield-shifted resonance is observed at high pH in the presence of enzyme (Figure 1A). As the pH is lowered, two peaks arise (Figure 1B); their chemical shifts are indicated by the open triangles in Figure 2. One of these (Figure 2, △) remains unaltered in chemical shift as pH is reduced to 5.5, while the other (Figure 2, ▽) titrates with an apparent pK of 5.9. The presence of two peaks suggests that isocitrate exists in two slowly exchanging forms. The chemical shift of the titrating form is upfield of the resonance of free isocitrate, implying that it is attributable not to the free compound but to a form bound to the enzyme with the α-carboxyl exposed to solvent.

The β-Carboxyl of Isocitrate. Figure 3 shows the pH dependence of the chemical shift of the β-carboxyl of isocitrate under various conditions. In the absence of isocitrate dehydrogenase, the shifts are almost identical with or without

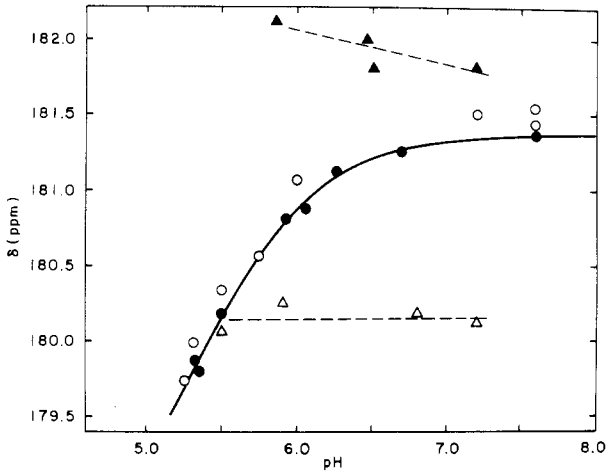


FIGURE 3: ^{13}C NMR chemical shifts of the β-carboxyl of isocitrate. Shifts were measured in the absence (○, ●) and presence of stoichiometric enzyme (▲, △). The filled symbols are shifts obtained with addition of 5 mM magnesium sulfate. The dashed lines are arbitrarily drawn to fit the data. The solid line is a fit to a titration curve with values given in Table I.

magnesium sulfate. The chemical shift at high pH for the unprotonated form is 0.14 ppm higher without magnesium, a difference not much greater than the 0.1 ppm error in peak position assignment. The data obtained without magnesium yield values of 178.22 ± 0.58 for the shift of the protonated group, 181.53 ± 0.05 for the shift of the unprotonated group, and a pK of 5.28 ± 0.14, which are close to the values (Table I) used in Figure 3 to construct the line.

When isocitrate is bound to the enzyme, a dramatic difference in the shifts in the presence and absence of magnesium is observed (compare parts C and D of Figure 1). In the presence of Mg^{2+} (▲), the carboxyl resonance is shifted downfield of the unprotonated resonance of isocitrate throughout the pH range covered, suggesting polarization of the charge on the β-carboxyl. In contrast, when magnesium is absent, the observed shift of enzyme-bound isocitrate is substantially upfield of the unprotonated resonance. This does not require protonation of the carboxyl but could arise from hydrogen bonding, ring-current effects or distortion of the carboxyl geometry (Palmer et al., 1982).

^{13}C Spectroscopy of the γ-Carboxyl of Isocitrate. Figure 4 shows the pH dependence of the resonance of the γ-carboxyl under the same conditions as in Figures 1–3. The pK observed for the compound in the absence of enzyme is similar to that obtained from titrations of the other carboxyls (Table I). This agreement in pK suggests that the observed shifts reflect a single titration event.

In the presence of isocitrate dehydrogenase (Figure 1E), an upfield shift is seen (δ = 180.7). This shift is slightly larger in the absence of magnesium. In both cases, the chemical shift of the enzyme-bound isocitrate resonance remains constant as the pH is changed throughout a range in which the resonance position of free isocitrate changes by about 0.8 ppm. Thus, the γ-carboxyl appears to remain in a constant environment on the enzyme.

Carbon-13 Resonance of the α-Carbon of Isocitrate. The α-carbon resonance of free isocitrate is found at 74.6 ppm in the high-pH unprotonated form. Since this is near the glycerol resonance at 72.9 ppm, NMR spectra were collected for enzyme samples that were dialyzed against buffer from which glycerol was omitted. No resonance (40 000 scans) was observed with stoichiometric concentrations of enzyme and isocitrate. With isocitrate in excess of enzyme (up to 2.5:1), no resonance was observed. Possibly, free isocitrate is in intermediate exchange with a broadened and/or greatly perturbed bound resonance. A carbon with attached protons is expected to have broad peaks due to incomplete averaging of contributions to relaxation arising from dipolar coupling or chemical shift anisotropy. Such broadening from dipolar contributions has been postulated for the methylene carbon of oxaloacetate bound to citrate synthase (Kurz et al., 1985).

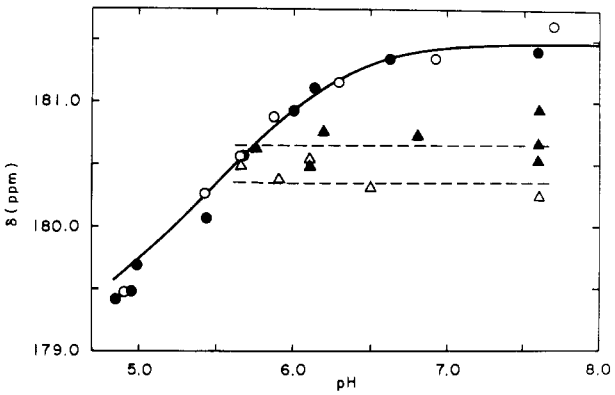


FIGURE 4: ^{13}C NMR chemical shifts of γ-carboxyl of isocitrate. Shifts were measured in the absence of enzyme (●, ○) and fit (solid line) with values given in Table I. Chemical shifts measured in the presence of enzyme are shown (▲, △). Filled symbols show data taken in the presence of 5 mM magnesium sulfate.

α-Ketoglutarate. The ^{13}C NMR resonances of α-ketoglutarate do not change when stoichiometric amounts of isocitrate dehydrogenase are added. The α-carboxyl remains unshifted at 170.7 ± 0.1 ppm throughout the pH range 5–8. The γ-carboxyl titrates with a pK of 4.76 from 178.5 for the protonated form to 182.1 for the ionized form. The carbonyl carbon has a resonance at 205.8 ppm. These values agree with those presented by Viswanathan et al. (1982), but the presence of the hydrated form could not be demonstrated at concentrations near the level of enzyme. Line widths of approximately 8 Hz are found for the α- and γ-carboxyls and 12 Hz for the α-carbonyl. No broadening occurs in complexes with enzyme using ligand-to-enzyme ratios from 1 to 9. In the absence of other data, one might conclude from the NMR results that α-ketoglutarate does not bind to isocitrate dehydrogenase under the conditions used; however, direct binding studies (Colman, 1969), proton relaxation measurements (Villafranca & Colman, 1974), and electron spin resonance (Levy & Villafranca, 1977) indicate that binding does occur. The absence of shifts or line broadening suggests either little polarization of the carbon–oxygen bonds in the enzyme–α-ketoglutarate complex and/or rapid exchange with free α-ketoglutarate.

Effect of NADPH on the Enzyme–α-Ketoglutarate Complex. In the presence of metal and NADPH, exchange of a proton on the β-carbon of α-ketoglutarate has been observed to be catalyzed by isocitrate dehydrogenase (Rose, 1960). When 1 mM NADPH was added to a 1:1 complex of enzyme and α-ketoglutarate at pH 7.5, the resonances of the α-carboxyl and the γ-carboxyl shifted to 182.2 and 183.4 ppm, respectively, with disappearance of the original peaks. These do not coincide with resonances of isocitrate. After the acquisition of the NMR spectra, the samples were assayed for the presence of α-ketoglutarate and were found to contain less than 10% of the original amount of α-ketoglutarate. Resonances at the same positions as those observed after incubation with NADPH (within 0.05 ppm) were found for α-ketoglutarate reduced with excess sodium borohydride. That these arise from α-hydroxyglutarate was verified by the NMR spectrum of the latter compound (Sigma). Thus isocitrate

dehydrogenase appears to catalyze the conversion of α-ketoglutarate to the alcohol in the absence of CO_2. This conversion was also followed spectrophotometrically by monitoring the loss of NADPH at 360 nm in the presence of isocitrate dehydrogenase, α-ketoglutarate, and magnesium. At 2.5 mM α-ketoglutarate, the rate was 0.004 μmol/(min·mg of enzyme) (pH 7.2) compared with 29 μmol/min·mg) for the reduction without carboxylation observed for the analogue bromoketoglutarate (pH 7.0), and 29 μmol/(min·mg) for the overall isocitrate dehydrogenase reaction (pH 7.2).

The ^{13}C NMR spectrum of the mixture of NADPH, enzyme, magnesium, and α-ketoglutarate enriched in the α-carbonyl must be that of an equilibrium mixture of reactants. When α-ketoglutarate enriched with ^{13}C in the carbonyl carbon was used, the carbonyl peak at 205 ppm disappeared. The resonance of the α-carbon of α-hydroxyglutarate is at 72.9 ppm. In addition, a broad peak ($\Delta\nu_{1/2} \approx 150$ Hz) appears at 124 ppm that is not found in other enzyme–ligand complexes. This peak could arise from the enolate form of α-ketoglutarate which from substituent rules (Brouwer & Stothers, 1972) would be expected at 126 ppm.

Discussion

When isocitrate is titrated between pH 5 and pH 8, the peaks of all three carboxyls shift downfield as the pH is decreased. The pK's obtained from all three titrations are approximately the same (Table I). The largest chemical shift upon protonation is that for the β-carboxyl group. Thus, the β-carboxyl group is the most probable locus for the final ionization of isocitrate. The shift upon ionization of 3.6 ppm is close to that observed for other carboxylic acids (Hagen & Roberts, 1969), and the shifts observed for the other carboxyls would result from substituent effects acting through chemical bonds (Grant & Paul, 1964). However, the possibility that the last ionization is not strictly confined to the β-carboxyl cannot be ruled out. Since the addition of magnesium has little effect on any of the carboxyl groups, the site of magnesium binding cannot be determined for free isocitrate.

When it is bound to isocitrate dehydrogenase, the carboxyl groups of isocitrate have no pH variation in ^{13}C chemical shifts, with the exception of a resonance observed for the α-carboxyl in the absence of metal, suggesting that all three carboxyls are ionized in the enzyme-bound complex. This could arise from positively charged group(s) in the substrate binding region. Arginine(s), modified by 2,3-butanedione, has (have) been postulated to be in this region (Ehrlich & Colman, 1977). The α- and γ-carboxyls are slightly deshielded (0.5–0.8 ppm) in the enzyme complex, compared with free ionized isocitrate. Magnesium decreases the magnitude of the deshieldings by about 0.3 ppm. In the absence of magnesium, the α-carboxyl resonance is split into two peaks (at a stoichiometry of 1:1, isocitrate to enzyme subunits). One peak shifts with pH with a pK of 5.96 ± 0.16. This may indicate a mode of binding in which this carboxyl group is accessible to solvent (the presence of an impurity is unlikely since a single peak is observed in the absence of enzyme). This could result from a structural asymmetry in the identical subunits of the isocitrate dehydrogenase dimer. Other evidence for asymmetric behavior of the two subunits of the $NADP^+$-dependent isocitrate dehydrogenase dimer is provided by the enhancement of enzyme fluorescence by $NADP^+$ at a stoichiometry of 0.5 site per peptide chain (Mas & Colman, 1985) and by the biphasic reaction at the nucleotide site of the enzyme with 2-[(4-bromo-2,3-dioxobutyl)thio]-1,N^6-ethenoadenosine 2′,5′-bisphosphate (Bailey & Colman, 1985).

The ^{13}C resonance of the β-carboxyl of isocitrate is shifted upfield by close to 1.5 ppm upon binding to the enzyme in the absence of magnsium (Figure 3). The position of this resonance does not change in the pH range 5.5–7.3. This shift is greater than that expected from local environmental effects or salt bridges (Palmer et al., 1982) but smaller than the shift produced by protonation (Table I). The additional shift may arise from alteration in the geometry of the carboxyl group or alteration of the partial charge on the α-hydroxyl carbon.

When magnesium is added to the enzyme–isocitrate complex, the resonance of the β-carboxyl is deshielded by about 2 ppm and the resonance is downfield of that of free isocitrate. Deshielding of the 4-carboxyl group of benzoic acid has been observed when this inhibitor is bound to carboxypeptidase A (Palmer et al., 1982). This deshielding has been attributed to interaction with a metal ion and a carboxyl side chain on the enzyme. For oxaloacetate binding to citrate synthase, increased shielding is observed and attributed to partial protonation of the adjacent carbonyl group (Kurz et al., 1985). Since the deshielding of the β-carboxyl of isocitrate is only observed with magnesium present, interaction with metal is probable. In addition, polarization of the OH bond could occur, resulting in a distribution of charge density. The resulting metal-dependent alteration of charge on the β-carboxyl could activate the subsequent metal-activated decarboxylation of this group from the oxalosuccinate intermediate.

Further insight into the charge rearrangements should be provided from changes in the ^{13}C resonance of the α-carbon. Unfortunately, this resonance could not be observed with 0.5 mM enzyme. Under the conditions used, a line width greater than 200 Hz could be unobservable. A line width of about 100 Hz is expected for an immobilized group experiencing dipole relaxation from a single attached proton [assuming a rotational correlation time of 5×10^{-5} s (Cantor & Schimmel, 1980)]. Additional line broadening could arise from chemical shift anisotropy.

For α-ketoglutarate, no shifts were observed in the presence of enzyme. Significant broadening in the presence of excess α-ketoglutarate was not observed. The lack of changes for α-ketoglutarate cannot be due to failure to bind, since the measured dissociation constants are 20 μM (Colman, 1969) in the absence of metal and about 300 μM from the manganese–enzyme complex (Villafranca & Colman, 1972).

Additional evidence for α-ketoglutarate binding was obtained from relaxation measurements (Villafranca & Colman, 1974). The current experiments suggest that α-ketoglutarate, when enzyme bound, remains in a configuration that differs little from its free configuration. The lack of anisotropy in the manganese electron paramagnetic resonance spectrum of the metal–enzyme-α-ketoglutarate complex is consistent with this conclusion (Levy & Villafranca, 1977).

The addition of NADPH might be expected to alter the electron distribution on α-ketoglutarate in the presence of enzyme and metal ion, since exchange of the β-hydrogen is catalyzed in this quaternary complex (Rose, 1960). Unfortunately, a competing reaction in which α-hydroxyglutarate is formed prevents observation of any effects. The spectrum of the product in the equilibrium mixture (in which the concentration of free α-ketoglutarate was too low to detect) is unshifted from that of free α-hydroxyglutarate. The broad peak centered at 124 ppm observed in this mixture may be attributed to the bound enol form of α-ketoglutarate. The broadening may be due to slow exchange with product and/or substrate. An enol form is not distinguished from the keto form in free α-ketoglutarate (Viswanathan et al., 1982; Cooper & Redfield, 1975). The enol form of α-ketoglutarate may be

stabilized when enzyme bound, which would facilitate the reductive carboxylation reaction by the mechanism proposed by Rose (1960).

The ^{13}C NMR studies have shown that isocitrate binds to isocitrate dehydrogenase with all carboxyls ionized, as predicted from kinetic and binding studies (Colman, 1983). They have indicated that metal significantly changes the electronic environment of the β-carboxyl. The alteration of the chemical shift of the β-carboxyl in the enzyme–metal–isocitrate complex and the failure to observe distinct shifts in either carboxyl in the enzyme–α-ketoglutarate complex suggest that the polarization of the β-carboxyl by the enzyme contributes to the tighter binding of isocitrate and is significant for the oxidative decarboxylation function of isocitrate dehydrogenase.

References

Bacon, C. R., Bednar, R. A., & Colman, R. F. (1981) *J. Biol. Chem. 256*, 6593–6599.

Bailey, J. M., & Colman, R. F. (1985) *Biochemistry 24*, 5367–5377.

Brouwer, H., & Stothers, J. B. (1972) *Can. J. Chem. 50*, 1361–1370.

Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry*, Vol. II, p 461, W. H. Freeman, San Francisco.

Colman, R. F. (1969) *Biochim. Biophys. Acta 191*, 469–472.

Colman, R. F. (1972a) *J. Biol. Chem. 247*, 215–223.

Colman, R. F. (1972b) *J. Biol. Chem. 247*, 6727–6729.

Colman, R. F. (1983) *Pept. Protein Rev. 1*, 41–69.

Cooper, A. J. L., & Redfield, A. G. (1975) *J. Biol. Chem. 250*, 527–532.

Ehrlich, R. S., & Colman, R. F. (1976) *Biochemistry 15*, 4034–4041.

Ehrlich, R. S., & Colman, R. F. (1977) *Biochemistry 16*, 3378–3383.

Ehrlich, R. S., & Colman, R. F. (1986) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 45*, 1648.

Grant, D. M., & Paul, E. G. (1964) *J. Am. Chem. Soc. 86*, 2984–2990.

Hagan, R., & Roberts, J. D. (1969) *J. Am. Chem. Soc. 91*, 4504–4506.

Hartman, F. C. (1981) *Biochemistry 20*, 894–898.

Johanson, R. A., & Colman, R. F. (1981) *Arch. Biochem. Biophys. 207*, 9–20.

Kurz, L. C., Ackerman, J. J. H., & Drysdale, G. R. (1985) *Biochemistry 24*, 452–457.

Levy, R. S., & Villafranca, J. J. (1977) *Biochemistry 16*, 3293–3301.

Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431–441.

Mas, M. T., & Colman, R. F. (1985) *Biochemistry 24*, 1634–1646.

Palmer, A. R., Ellis, P. D., & Wolfenden, R. (1982) *Biochemistry 21*, 5056–5059.

Rose, Z. B. (1960) *J. Biol. Chem. 235*, 928–933.

Villafranca, J. J., & Colman, R. F. (1972) *J. Biol. Chem. 247*, 209–214.

Villafranca, J. J., & Colman, R. F. (1974) *Biochemistry 13*, 1152–1160.

Viswanathan, T. S., Johnson, R. E., & Fisher, H. F. (1982) *Biochemistry 21*, 339–345.

Photoaffinity Labeling of Rabbit Muscle Fructose-1,6-bisphosphate Aldolase with 8-Azido-1,N^6-ethenoadenosine 5′-Triphosphate†

Krzysztof Palczewski* and Marian Kochman

Division of Biochemistry, Institute of Organic and Physical Chemistry, Technical University of Wrocław, 50-370 Wrocław, Poland

Received November 5, 1986; Revised Manuscript Received February 5, 1987

ABSTRACT: Steady-state kinetic measurements have shown that 8-azido-1,N^6-ethenoadenosine 5′-triphosphate (8-N_3-ϵATP) can be noncovalently bound to rabbit muscle fructose 1,6-bisphosphate aldolase with K_i = 0.075 mM at pH 8.5. This binding is purely competitive with substrate and occurs at the strong binding site for mononucleotides. Photoaffinity labeling of aldolase in the presence of 8-azido-1,N^6-ethenoadenosine 5′-triphosphate results in inactivation of the enzyme. Aldolase is protected against modification in the presence of the inhibitors hexitol 1,6-bisphosphate or ATP. The labeling is saturable, and a good correlation is observed between the loss of enzymatic activity and the incorporation of 8-N_3-ϵATP into aldolase. In addition, aldolase loses its ability to bind to phosphocellulose following modification. Digestion of labeled protein with trypsin, chymotrypsin, and cyanogen bromide revealed substantial modification of peptide 259–269. Thr-265 was identified as the residue that was covalently modified by 8-N_3-ϵATP. On the basis of these results and other data we propose a model for the mononucleotide binding site.

The inhibitory effect of adenine nucleotides on aldolases A and B was discovered by Spolter and co-workers over twenty years ago (Spolter et al., 1965). Recently our knowledge concerning interaction of these physiologically important molecules with aldolase has been extended. It has been shown that each subunit of the aldolase tetramer has one strong and one weak binding site for mononucleotides (Kasprzak & Kochman, 1980a). The electronegative phosphate group of the nucleotide plays a predominant role in binding to the muscle enzyme. A secondary role has been ascribed to the adenosine moiety (Kasprzak & Kochman, 1980a). It has been found that binding to the weak site can be markedly reduced

† This study has been partially supported by Polish Academy of Science Grant CPBR 3.13.4.

* Address correspondence to this author at the Department of Ophthalmology, University of Florida College of Medicine, J. Hillis Miller Health Center, Gainesville, FL 32610.

0006-2960/87/0426-3466$01.50/0