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Interaction of Isocitrate Dehydrogenase with (*RS*)-3-Bromo-2-ketoglutarate. A Potential Affinity Label for α-Ketoglutarate Binding Sites†

Fred C. Hartman

ABSTRACT: The interaction of oxidized nicotine adenine dinucleotide phosphate dependent isocitrate dehydrogenase (from pig heart) with (*RS*)-3-bromo-2-ketoglutarate was investigated in an effort to evaluate the reagent's potential as a selective reagent for α-ketoglutarate binding sites. The enzyme is rapidly inactivated by 0.1 mM bromoketoglutarate at pH 7.4. With increasing concentrations of reagent, the reaction shows a rate saturation; the minimum inactivation half-time is 3 min and K_{inact} for bromoketoglutarate is 250 μM. Isocitrate and $NADP^+$ protect against inactivation, while ketoglutarate does not. When tested in the assay that monitors isocitrate oxidation, bromoketoglutarate is a competitive inhibitor (K_i = 100 μM) of the dehydrogenase. As judged by oxidation of NADPH, bromoketoglutarate is also a substrate for isocitrate dehydrogenase, exhibiting a K_m of 250 μM and a V_{max} comparable to that for isocitrate oxidation. The reduction of bromoketoglutarate is competitively inhibited by isocitrate (K_i = 3 μM) and ketoglutarate (K_i = 50 μM). Like the enzyme-catalyzed oxidation of isocitrate, the reduction of bromoketoglutarate is stereospecific, requires divalent metal ions, and shows absolute specificity for NADPH. However, since CO_2 is not required for catalytic turnover of bromoketoglutarate, its reduction is likely comparable to that of oxalosuccinate rather than the reductive carboxylation of ketoglutarate. Although bromoketoglutarate, as a substrate for isocitrate dehydrogenase, clearly has affinity for the active site, the irreversible inactivation of the enzyme by the reagent may result from modification outside the active-site region, since inactivation during catalytic turnover of bromoketoglutarate is not observed. Commercial isocitrate dehydrogenase is purified 12-fold by affinity chromatography on thiol–agarose alkylated by bromoketoglutarate.

α-Ketoglutarate is an unusually diverse metabolite: a Krebs cycle intermediate, a critical link between carbohydrate and amino acid metabolism as the amino group acceptor in numerous transaminations, a component of the malate–aspartate shuttle which accomplishes net transport of NADH into mitochondria, and a biosynthetic precursor of vitamin K, lysine (in fungi), and porphyrins (in plants). Because of the large number of enzymes which thus possess binding sites for α-ketoglutarate, the design of reactive analogues of α-ketoglutarate as potential chemical probes of these sites appears warranted. One such reagent, (*RS*)-3-bromo-2-ketoglutarate, was prepared and shown to inactivate glutamate synthase (Mäntsälä & Zalkin, 1976). For further exploration of its

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effectiveness as an α-ketoglutarate analogue, bromoketoglutarate has now been tested as an active-site reagent for $NADP^+$-dependent isocitrate dehydrogenase [threo-D_s-isocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] from pig heart. This well-characterized, commercially available enzyme was chosen as a model system because of mechanistic considerations. During the reductive carboxylation of α-ketoglutarate to form isocitrate, the initial catalytic event is stereospecific proton abstraction from C-3 (Rose, 1960). By the placement of a good leaving group (i.e., a bromine atom) at C-3 of α-ketoglutarate, it was hoped that the acid–base group that effects proton abstraction would be alkylated and its identity thereby revealed. Bromopyruvate, an active-site reagent first introduced by Meloche (1967) and chemically similar to bromoketoglutarate, has proven to be quite versatile in labeling pyruvate binding sites (reviewed by Hartman, 1977). As one example, the pyruvate binding site of 2-keto-3-deoxy-6-phosphogluconate aldolase is selectively modified by bromopyruvate via esterification of the glutamyl α-carboxylate which participates as the catalytically functional base (Meloche, 1973).

I describe here the properties of bromoketoglutarate as both inactivator and substrate for isocitrate dehydrogenase and illustrate the use of bromoketoglutarate in the preparation of an effective affinity adsorbent for the enzyme.

Experimental Procedures

Materials

Isocitrate dehydrogenase, DL-isocitrate, NADP, NADPH, α-ketoglutarate, *N*-acetylhomocysteine thiolactone, Hepes,[1] Nbs_2, and glutathione were purchased from Sigma Chemical Co. Affi-Gel 102 (an aminoalkyl-substituted agarose) was a product of Bio-Rad Laboratories. All other reagents were of the highest purity commercially available.

Methods

Elemental Analyses. Analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.

Electrophoresis. Polyacrylamide gel electrophoresis was carried out at pH 8.6 on standard 7% gels with an analytical apparatus from Canal Industrial Corp. according to the manufacturer's instructions. Gels were stained with Amido-Schwarz and destained electrophoretically. $NaDodSO_4$–polyacrylamide electrophoresis was performed as described by Weber & Osborn (1969). Protein was visualized by staining gels with Coomassie blue.

Isocitrate Dehydrogenase Activity. Isocitrate oxidation catalyzed by the dehydrogenase was assayed at 25 °C by monitoring NADPH appearance spectrophotometrically at 340 nm. Conditions were identical with those described earlier (Colman, 1968). The enzyme's requirement for divalent metal ions was provided by Mn^{2+}. A Beckman Acta V recording spectrophotometer equipped with a thermostated sample compartment was used for all assays.

Inactivation Studies. Commercial isocitrate dehydrogenase (sp act. 1.4 units/mg), provided in 50% aqueous glycerol, was dialyzed at 4 °C against 0.05 M Hepes/0.2 M NaCl/1 mM EDTA/20% glycerol (pH 7.4). The dialyzed solution of protein (7 mg/mL), in which the dehydrogenase was stable indefinitely at 4 °C, served as the stock for subsequent modification experiments. Aliquots from the stock were diluted 1:10 into 0.04 M triethanolamine hydrochloride (pH 7.4). Modifications were initiated by the addition of bromoketoglutaric acid to the diluted stock solutions at 25 °C; periodically, 20 μL-aliquots were withdrawn and assayed in 1-mL reaction cuvettes for dehydrogenase activity.

Synthesis of (RS)-3-Bromo-2-ketoglutaric Acid. To an efficiently stirred solution of α-ketoglutaric acid (10 g, 68.5 mmol) in 20 mL of glacial acetic acid at 50 °C was added 11 g (3.55 mL, 68.8 mmol) of bromine in 100-μL portions during 2 h. The reaction mixture was extracted three times with 100-mL portions of cyclohexane to remove acetic acid and HBr formed during the bromination. The residual bromoketoglutaric acid was placed in a shallow crystallizing dish and desiccated over moist KOH pellets and Drierite for several days, during which time the bromoketoglutaric acid (10.8 g, 70%) solidified. Paper chromatography (Whatman 1) with BuOH/HOAc/H_2O (7:2:5 v/v) as solvent revealed the complete absence of α-ketoglutaric acid (R_f 0.75), detectable with $AgNO_3$ (Trevelyan et al., 1950) or dinitrophenylhydrazine (Gray, 1952) sprays, and the presence of a single new component (R_f 0.84), detectable with an Nbs spray for reactive halogen compounds (Schloss & Hartman, 1977) in addition to its visualization with the ketone reagents. Anal. Calcd for $C_5H_5BrO_5$: C, 26.69; H, 2.24; Br, 35.52. Found: C, 26.58; H, 2.35; Br, 35.44.

Aqueous solutions of bromoketoglutaric acid (the free-acid form) could be stored frozen for several weeks without noticeable change. Bromoketoglutaric acid was assyed by its reaction with glutathione. To 0.9 mL of 2 mM glutathione in 0.05 M Hepes/1 mM EDTA (pH 7.5) was added 0.1 mL of 10 mM (by weight) bromoketoglutaric acid in water. The decrease in sulfhydryl concentration was followed by the procedure of Ellman (1959). Aliquots (0.1 mL) of the glutathione/bromoketoglutarate reaction mixture were added to cuvettes that contained 2.4 mL of 0.4 mM Nbs_2/0.1 M $NaHCO_3$/1 mM EDTA (pH 8.1); the sulfhydryl concentration was determined from the A_{412nm} (ε 13 600). On the basis of these measurements, the alkylation of glutathione's sulfhydryl group by bromoketoglutarate was completed within 5 min, and the calculated concentration of bromoketoglutaric acid in the stock solution was 9.8 ± 0.4 mM.

Preparation of Affinity Adsorbent for Isocitrate Dehydrogenase. Affi-Gel 102 (10 mL of wet gel with a specified capacity of 16 μmol/mL) was subjected to thiolation with *N*-acetylhomocysteine thiolactone (Cuatrecasas, 1970); the product contained 16.6 ± 0.8 μmol of sulfhydryl/mL of wet gel as assayed with Nbs_2 (Cuatrecasas, 1970). Thiolated gel (5 mL) was suspended in 5 mL of 0.1 M $NaHCO_3$ (pH 8.1) and gently stirred during the following treatment with 0.01 M bromoketoglutarate at room temperature for 15 min. At this time, a negative response with Nbs_2 showed that the alkylation was completed. The alkylated gel was then collected by suction filtration, washed exhaustively with water, and resuspended in water for storage at 4 °C.

Results

Incubation of isocitrate dehydrogenase with (*RS*)-3-bromo-2-ketoglutarate results in rapid loss of enzymic activity (Figure 1). Although the initial rate of modification obeys pseudo-first-order kinetics, deviation from such clearly occurs beyond 50–70% inactivation. Less than 1% of the initial activity remains after a 3-h exposure to 1 mM bromoketoglutarate. Activity is not restored by dialysis, gel filtration, or treatment with thiols. $NADP^+$ and isocitrate afford a high degree of protection of isocitrate dehydrogenase against inactivation by bromoketoglutarate, $MnCl_2$ affords significant

[1] Abbreviations used: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Nbs, 5-thio-2-nitrobenzoic acid; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

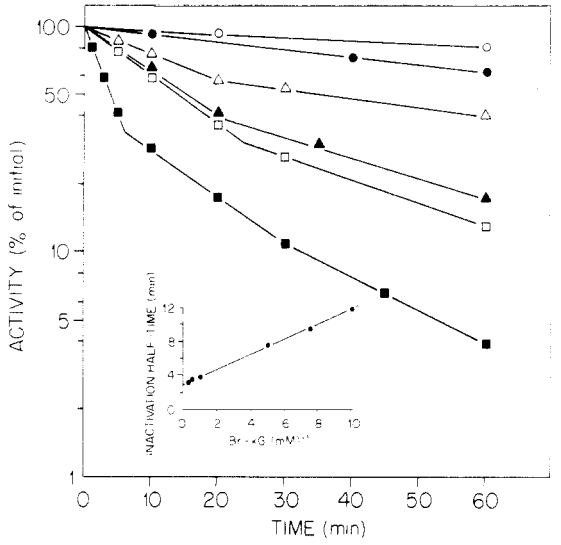

FIGURE 1: Inactivation of isocitrate dehydrogenase by 1.0 mM (■) and 0.1 (□) bromoketoglutarate (Br-kG) in the absence of other ligands and by 0.1 mM bromoketoglutarate in the presence of 1 mM α-ketoglutarate (▲), 0.3 mM $MnCl_2$ (△), 1 mM DL-isocitrate (●), or 0.1 mM NADP (○). Dependency of inactivation rate of isocitrate dehydrogenase on the reciprocal of bromoketoglutarate (Br-kG) concentration is shown in the inset. For details, see Experimental Procedures.

protection, while α-ketoglutarate provides little, if any, protection (Figure 1). A rate–saturation effect is observed upon plotting the inactivation half-time (measured from initial rates of inactivation) as a function of reciprocal reagent concentration. From the data illustrated in Figure 1 (inset), the calculated K_{inact} (the reagent concentration yielding one-half the maximal rate of inactivation) for bromoketoglutarate is 250 μM;[2] the minimal half-time is 3 min, which corresponds to a k (pseudo-first-order rate constant at infinite reagent concentration) of 0.0039 s^{-1}.

As judged by the oxidation of NADPH, bromoketoglutarate is a substrate for isocitrate dehydrogenase. In 0.04 M triethanolamine hydrochloride (pH 7.4) containing 0.1 mM NADPH and 2 mM $MnCl_2$ (the same assay system as used for isocitrate oxidation with substitution of reduced nucleotide for oxidized), the specific activity of the impure, commercial dehydrogenase with 1 mM bromoketoglutarate is 1.4 units/mg—the same value as determined with isocitrate and $NADP^+$ as substrates. When the assay solution contains a molar excess of NADPH, only 45–48% of the total bromoketoglutarate (quantitated in the concentrated stock solution by reaction with glutathione as described above) is reduced. NADPH oxidation with bromoketoglutarate as cosubstrate does not require CO_2 (assay solutions were sparged with N_2 before initiation of reaction by introduction of enzyme) but is absolutely dependent on divalent metal ions. NADH cannot replace NADPH. Kinetic parameters for bromoketoglutarate were determined with homogeneous isocitrate dehydrogenase (purified as described below) having a specific activity (isocitrate as substrate) of 27 units/mg [a value of 29 was reported by Colman (1968)]. Bromoketoglutarate exhibits a K_m of 250 μM and a V_{max} of 29 units/mg; the reduction of bromoketoglutarate is inhibited in a linearly competitive fashion by both D-isocitrate (K_i = 3 μM) and α-ketoglutarate (K_i = 50 μM) (Figure

[2] The various kinetic parameters of the interaction of bromoketoglutarate with isocitrate dehydrogenase are expressed in terms of the total chemical concentration of bromoketoglutarate, since it is not known whether the R, S, or both optical isomers are active in the given effect under study.

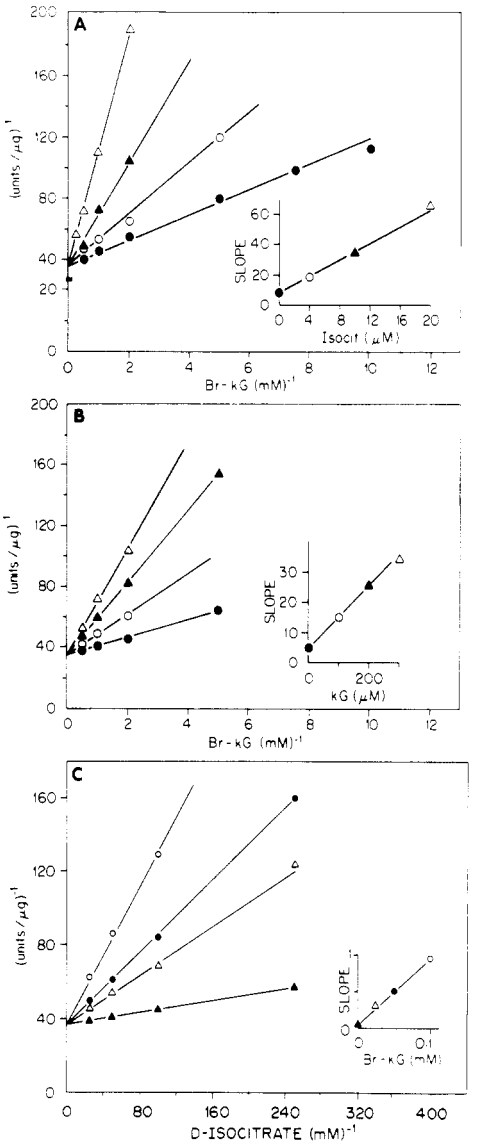

FIGURE 2: (A) Initial velocity patterns for the reduction of bromoketoglutarate (Br-kG) by isocitrate dehydrogenase (●) and competitive inhibition by D-isocitrate at concentrations of 4 μM (○), 10 μM (▲), and 20 μM (△). All reaction mixtures contained 0.1 mM NADPH and 1.77 μg of the dehydrogenase. (B) Initial velocity patterns for the reduction of bromoketoglutarate (Br-kG) by isocitrate dehydrogenase (●) and competitive inhibition by α-ketoglutarate at concentrations of 100 μM (○), 200 μM (▲), and 300 μM (△). Other conditions are as indicated in (A). (C) Initial velocity patterns for the oxidation of isocitrate by dehydrogenase (▲) and competitive inhibition by bromoketoglutarate (Br-kG) at concentrations of 25 μM (△), 50 μM (●), and 100 μM (○). All reaction mixtures contained 0.1 mM NADP and 0.177 μg of the dehydrogenase. Secondary plots are shown in the inset. Other details are given under Experimental Procedures and Results.

2A,B). Dissociation constants for D-isocitrate and α-ketoglutarate have been reported as 1–3 μM and 59–290 μM, respectively (Villafranca & Colman, 1972; Uhr et al., 1974). As shown in Figure 2C, bromoketoglutarate is also a competitive inhibitor (K_i = 100 μM) of isocitrate dehydrogenase with D-isocitrate as substrate (K_m = 2 μM, V_{max} = 28 units/mg).

Commercial isocitrate dehydrogenase was purified by affinity chromatography on thiol–agarose alkylated by bromoketoglutarate. A 35-mL solution containing 200 mg of the dehydrogenase (sp act. 1.4 units/mg) in 0.02 M Hepes/0.1 mM EDTA/20% glycerol (pH 7.0) was applied at a flow rate of 18 mL/h to a 1.8-mL column (a Pasteur pipet) of the

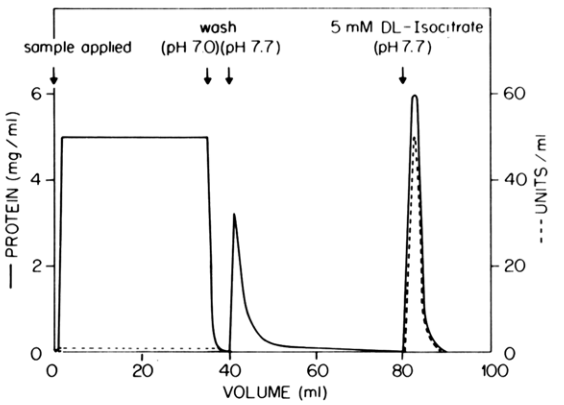

FIGURE 3: Affinity chromatography of isocitrate dehydrogenase on thiol–agarose alkylated by bromoketoglutarate. For details see Results.

derivatized agarose equilibrated with the same Hepes buffer. Of the 295 units applied to the column, only 25 were not retained. After sample application was completed, the column was eluted with equilibration buffer (about 10 mL) until the effluent was free of protein. The column was then washed with 40 mL of 0.02 M Hepes/0.1 mM EDTA/20% glycerol (pH 7.7) followed by elution with 10 mL of 0.01 M Hepes/5 mM DL-isocitrate/0.1 mM EDTA/20% glycerol (pH 7.7). The dehydrogenase was recovered in 85% yield (252 units with sp act. 16) from the isocitrate eluate (Figure 3). After dialysis of isocitrate dehydrogenase against the 0.02 M Hepes buffer (pH 7.0) to remove isocitrate, the enzyme was applied to a 1.8-mL column of carboxymethylcellulose equilibrated with dialysis buffer. The column was eluted with an 80-mL linear gradient of NaCl (0–0.08 M) dissolved in equilibration buffer. Dehydrogenase eluted at 0.06 M NaCl and was recovered in 90% yield (230 units with sp act. 28 units/mg). The high degree of purity of this preparation in contrast to the commercial material is demonstrated by the analytical gels shown in Figure 4. Chromatography of isocitrate dehydrogenase on carboxymethylcellulose, under somewhat different conditions than those described here, has been described (Colman, 1968; Cleland et al., 1969; Ehrlich & Colman, 1976).

Discussion

$NADP^+$-linked isocitrate dehydrogenase has a very stringent substrate specificity. Of the four stereoisomers of isocitrate, only the α-D$_s$,β-L$_s$ is a substrate. The only synthetic substrate for the enzyme described to date that clearly undergoes oxidative decarboxylation, analogously to D-isocitrate, is D-ġarcinia acid (an isocitrate analogue in which the β hydrogen is formally replaced by a hydroxyl group) (Plaut et al., 1975). In the reverse direction, the reduction of oxaloacetate (which can be considered an analogue of oxalosuccinate, the intermediate in the oxidative decarboxylation of isocitrate) to D-malate is catalyzed by pig liver $NADP^+$-linked isocitrate dehydrogenase (Illingworth & Tipton, 1970). A carboxyl group at the position corresponding to β in isocitrate has been considered an absolute requirement for activity as a substrate. Thus, it is surprising to find that (*RS*)-3-bromo-2-ketoglutarate is an excellent substrate for isocitrate dehydrogenase in the direction of reduction. However, there is no question that the activity observed is attributable to isocitrate dehydrogenase and not to some other enzyme present as a contaminant. Both isocitrate and α-ketoglutarate competitively inhibit the reduction of bromoketoglutarate, and their observed K_i values are quite similar to previously determined K_d values with isocitrate dehydrogenase (Villafranca & Colman, 1972; Uhr et al., 1974). The ratio of V_{max} for bromoketoglutarate reduction to V_{max} for isocitrate oxidation remains constant at 1 whether crude commercial enzyme or highly purified enzyme is used. Furthermore, as with natural substrate, the activity with bromoketoglutarate has an absolute requirement for divalent metal ions and NADPH.

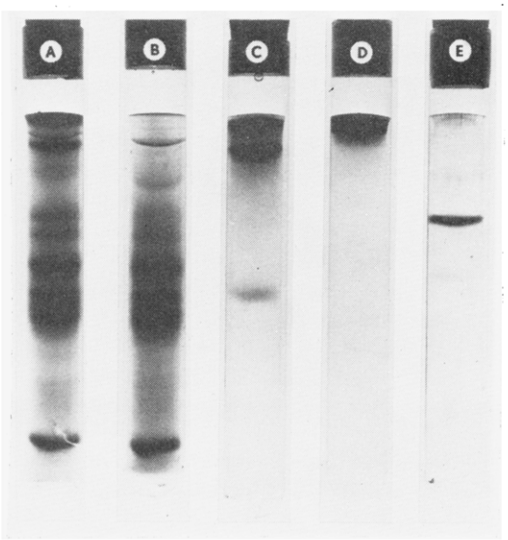

FIGURE 4: Gel electrophoresis of isocitrate dehydrogenase. Native gels of (A) commercial enzyme, 200 μg; (B) protein not adsorbed by affinity column, 200 μg; (C) isocitrate eluate from affinity column, 50 μg; and (D) isocitrate eluate after chromatography on carboxymethylcellulose, 50 μg. $NaDodSO_4$ gel (E) of sample shown in (D), 5 μg. Direction of migration in each gel is toward the anode (bottom of gel).

Unlike α-ketoglutarate, bromoketoglutarate is not carboxylated prior to reduction since the exclusion of CO_2 from the reaction mixture does not diminish the rate of reduction. Presumably, bromoketoglutarate is recognized by the enzyme as an analogue of oxalosuccinate and simply reduced to 2-hydroxy-3-bromoketoglutarate. The bromine atom of bromoketoglutarate can apparently occupy the active-site pocket that accommodates the β-carboxylate of oxalosuccinate. Only one of the two optical isomers of bromoketoglutarate is handled by the enzyme based on the oxidation of about 0.5 mol of NADPH/mol of (*RS*)-reagent. Accepting this likely stereospecifity, the equilibrium lies far in the direction of reduction, as is seen with oxaloacetate as substrate (Illingworth & Tipton, 1970).

Although the product of bromoketoglutarate reduction has not been characterized, $NADP^+$ is the coproduct from concomitant NADPH oxidation. This verification was made by the dehydrogenase-catalyzed oxidation of NADPH with an excess of bromoketoglutarate. Upon completion of the reaction, reduction of $NADP^+$ back to NADPH occurred upon adding excess isocitrate to the same reaction cuvette (data not shown).

The equivalence of the V_{max} for isocitrate oxidation and the V_{max} for bromoketoglutarate reduction is striking, especially considering the chemical differences in the two substrates and the fact that an oxidation and a reduction are being compared at the same pH. One possible explanation for this occurrence is that in both reactions dissociations of the nucleotide product from the enzyme are rate limiting and equal in rate. Isotope exchange (Uhr et al., 1974) and isotope effect (O'Leary & Limburg, 1977) studies demonstrate that product release is rate limiting in both the forward and reverse direction with natural substrates. However, with isocitrate and α-ketoglutarate as substrates, NADPH appeared to dissociate more rapidly from enzyme than did $NADP^+$ (Uhr et al., 1974), consistent with a V_{max} greater for isocitrate oxidation than for

ketoglutarate reductive carboxylation (Siebert et al., 1957). Irrespective of the reason for identical V_{max} values for isocitrate oxidation and bromoketoglutarate reduction, the synthetic substrate (given its high V_{max} and low K_m) is ideally suited for dissecting the oxidation–reduction partial reaction from the total reaction catalyzed by isocitrate dehydrogenase.

Despite the uncontestable interaction of bromoketoglutarate with the active site of isocitrate dehydrogenase, the irreversible inactivation may not be due to active-site modification for the following reasons. Insignificant protection against inactivation is provided by α-ketoglutarate even in the presence of high levels of CO_2. $NADP^+$ and $MnCl_2$ do provide substantial protection but clearly should not prevent reversible binding of bromoketoglutarate to the active site by direct competition. Inactivation is not observed during turnover of bromoketoglutarate as substrate (data not shown). However, given the fact that enzyme-catalyzed reduction of bromoketoglutarate proceeds with a k of 25 s^{-1} and that the maximal k for enzyme inactivation is 0.0039 s^{-1}, inactivation would not be expected during reduction of bromoketoglutarate unless this catalytic reaction is reversible. The degree of reversibility has not yet been determined.

The protective effect of isocitrate and the rate saturation with respect to bromoketoglutarate concentration, both consistent with an active-site modification, can be explained in other ways. Isocitrate may either render a given residue inaccessible to bromoketoglutarate through an induced conformational change or alternatively may prevent a conformational change subsequent to the enzyme's modification. The latter possibility appears excluded by the finding that the protected enzyme, subsequent to treatment with bromoketoglutarate, does not lose activity during removal of isocitrate by dialysis (data not shown). The rate–saturation effect could reflect either reversible binding of reagent to a secondary site or a rate-determining conformational change brought about by the chemical modification.

If the inactivation is not a consequence of prior reversible complex formation between reagent and active site of the enzyme, it is curious that the K_{inact}, K_m, and K_i for bromoketoglutarate are all in the range 100–250 μM. Perhaps the modification does involve the active site, and the lack of inactivation during catalytic turnover of bromoketoglutarate reflects an altered topology within the substrate binding site of the quaternary complex, enzyme·Mn^{2+}·NADPH·bromoketoglutarate, as compared to the binary complex, enzyme·bromoketoglutarate. However, both isocitrate and α-ketoglutarate bind to free enzyme, since isocitrate dehydrogenase obeys a random mechanism (Uhr et al., 1974); binding of these substrates to free enzyme is observed by direct measurements (Colman, 1969). Thus, the lack of protection by α-ketoglutarate against inactivation by bromoketoglutarate would still represent an anomaly with respect to a presumed active-site modification.

The affinity column described herein provides a quick, effective means of cleaning up rather crude commercial isocitrate dehydrogenase. Although the maximal capacity of the column has not been determined, the 1.8-mL column used permits the processing of 200 mg of crude enzyme in 5 h. A 12-fold enrichment is achieved, and the yellowish brown pigmentation in the starting material is completely eliminated.

Since bromoketoglutarate is sufficiently close in structure to α-ketoglutarate to be recognized as a substrate by isocitrate dehydrogenase, the reagent may prove useful as an active-site probe for some of the many enzymes that process α-ketoglutrate.

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