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3-Bromo-2-ketoglutarate: A Substrate and Affinity Label for Diphosphopyridine Nucleotide Dependent Isocitrate Dehydrogenase†

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ABSTRACT: Pig heart DPN-dependent isocitrate dehydrogenase is progressively inactivated by (*RS*)-3-bromo-2-ketoglutarate at pH 6.15 in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) buffer containing 2 mM $MnSO_4$ and 20% glycerol. With increasing concentrations of bromoketoglutarate, the reaction exhibits rate saturation: the minimum inactivation half-time is 20 min, with a K_{inact} of 6.2 mM. Isocitrate protects against inactivation to an extent consistent with its directly determined binding constant. 2-Ketoglutarate offers no significant protection, but it does not bind, competitively with isocitrate, to free enzyme. Only weak protection against inactivation is seen with the coenzyme DPN. The allosteric activator ADP offers protection against inactivation consistent with its directly determined binding constant. Incubation of bromoketoglutarate with the enzyme results in a loss of allosteric ADP activation at the same rate as inactivation. Equal protection is afforded by isocitrate or ADP against loss of activity and loss of ADP activation, making it likely that both processes result from the same molecular event. The measured loss of ADP activation must result from a change in the influence of ADP on the catalytic activity of unmodified subunits, suggesting that there is interaction between modified and unmodified subunits. The inactivation correlates with the covalent incorporation of approximately 0.83 mol of reagent/mol of average subunit, implying that three to four of the structurally distinct subunits of the enzyme tetramer must be modified for complete loss of activity. One-half of (*RS*)-3-bromo-2-ketoglutarate is also a substrate for isocitrate dehydrogenase with a K_m of 5 mM. The product is probably 2-hydroxy-2-bromoglutarate, since there is oxidation of DPNH, no requirement for CO_2, and no release of Br^-. The agreement of K_m and K_{inact} for bromoketoglutarate suggests that the active site is the target of modification. These results indicate that 3-bromo-2-ketoglutarate functions as an affinity label of the substrate binding site of the DPN-dependent isocitrate dehydrogenase.

A continuing series of investigations have been directed at the identification of residues that are involved in the active site of pig heart DPN-dependent isocitrate dehydrogenase [*threo*-D_s-isocitrate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.41] by chemical modification. By the use of a variety of "group-specific" reagents, several amino acid residues have been determined to be critical to the function of isocitrate dehydrogenase: lysyl (Shen & Colman, 1975; Hayman & Colman, 1977), cysteinyl (Mauck & Colman, 1976), argininyl (Hayman & Colman, 1978), and glutamyl or aspartyl residues (Ramachandran & Colman, 1977).

Results of studies with group-specific reagents are frequently difficult to interpret because of reaction with more than one amino acid residue. Affinity labeling offers a viable solution to this problem. An affinity label, or an active-site-directed reagent, is structurally analogous to the substrate (and, hence, can bind to the active site) but has in addition a functional group capable of reacting irreversibly with an amino acid

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residue within the active site. The specificity introduced by the binding interaction can result in rate enhancements as large as 10^{10}–10^{13} (Jencks, 1980) and makes it possible to identify amino acid residues within the active site.

Bromopyruvate, an affinity label first introduced by Meloche (1965), has proven to be quite versatile in labeling pyruvate binding sites (Hartman, 1977). Bromopyruvate has been shown to selectively modify the pyruvate binding site of 2-keto-3-deoxy-6-phosphogluconate aldolase by esterification of the glutamyl γ-carboxylate which participates as the catalytically functional base (Meloche, 1965, 1967, 1970, 1973; Meloche et al., 1972). Here we use 3-bromo-2-ketoglutarate, a 2-ketoglutarate analogue, which is chemically similar to bromopyruvate, as a means of identifying a general base in the isocitrate dehydrogenase mechanism. An amino acid residue might function as a general base to remove a C-3 proton from 2-ketoglutarate to form the enzyme-bound enolate form of ketoglutarate; the enolate could then add CO_2 to form oxalosuccinate. This same residue could function as a general acid to protonate the carbonyl oxygen of oxalosuccinate as it is reduced to isocitrate. The base on the enzyme might be sufficiently nucleophilic to displace a bromide ion from the C-3 carbon of BrKG and thereby form a covalent linkage between the enzyme and the substrate analogue. This alkylation reaction could provide the means for revealing the identity of a catalytically functional base. 3-Bromo-2-ketoglutarate was first prepared by Mäntsälä & Zalkin (1976) and shown to inactivate glutamate synthase. Recently, Hartman (1981) has shown that 3-bromo-2-ketoglutarate is both an inactivator and a substrate for the TPN-selective isocitrate dehydrogenase.

In this paper, 3-bromo-2-ketoglutarate is shown to inactivate the DPN-dependent isocitrate dehydrogenase from pig heart and to exhibit many of the characteristics of an affinity label. The reaction of 3-bromo-2-ketoglutarate as a substrate of the DPN–isocitrate dehydrogenase is evaluated. Measurement of the intrinsic reactivity of 3-bromo-2-ketoglutarate is made, and its implication for the usefulness of this compound as an affinity label is described. The following paper (Bednar et al., 1982) details the decomposition of BrKG and describes the identification of the modified residue. A preliminary account of this work has been presented (Bednar et al., 1981).

Experimental Procedures

Materials. DL-Isocitrate, DPN, DPNH, ADP, 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2),[1] 2-ketoglutarate, iodoacetate, Mes, dithiothreitol, reduced glutathione, Sephadex G-50 (80 mesh), and triethanolamine were purchased from Sigma Chemical Co. Manganese sulfate and EDTA were obtained from Mallinckrodt Chemical Co. Sodium borohydride and sodium dodecyl sulfate were products of Fisher Scientific and Pierce Chemical Co., respectively. Yeast alcohol dehydrogenase was obtained from Boehringer Mannheim Corp. Uniformly labeled 2-[^{14}C]ketoglutarate (0.25 mCi in 0.135 mg; calculated specific activity 600 dpm/pmol) dissolved in 2.5 mL of 0.1 N HCl was obtained from New England Nuclear. [1-^{3}H]Ethanol, a gift from Dr. Don Dennis, was originally obtained from New England Nuclear Corp. ACS counting cocktail was purchased from Amersham Corp.

Preparation of (RS)-3-Bromo-2-ketoglutaric Acid. (*RS*)-3-Bromo-2-ketoglutaric acid (BrKG) was synthesized by direct bromination of 2-ketoglutarate (2-KG) as described by Hartman (1981). Radioactive 3-bromo-2-[^{14}C]ketoglutaric acid was prepared on a smaller scale by using uniformly labeled 2-[^{14}C]ketoglutaric acid. To the labeled 2-ketoglutarate in 2.5 mL of 0.1 N HCl was added 40 mg (0.27 mmol) of unlabeled 2-ketoglutarate, and the resulting solution was lyophilized to dryness. The thoroughly dried residue was dissolved in 100 μL of glacial acetic acid, and following the addition of 15 μL (0.30 mmol) of bromine, the reaction mixture was incubated for 2 h at 50 °C. At this time, the reaction mixture was desiccated at 25 °C over Drierite and moist pellets of KOH; after 2 days, the bromoketoglutarate (46 mg, 75%) solidified. This product appeared homogeneous by paper chromatography (Hartman, 1981).

Determination of the Specific Radioactivity of 3-Bromo-2-[^{14}C]ketoglutarate. The method described is essentially that of Norton et al. (1975) for ascertaining the specific activity of $NaB^{3}H_4$ by the reduction of glutathione whose sulfhydryl group was alkylated by 3-chloroacetol phosphate. A solution (1 mL) containing 30 μmol of glutathione and 20 μmol of bromoketoglutarate in 0.2 M sodium bicarbonate (pH 8.0) was incubated at 25 °C for 30 min and then placed in an ice bath. Sodium borohydride (3.8 mg) was added to the glutathione/bromoketoglutarate reaction mixture, and after the solution had remained on ice for 1 h, its pH was adjusted to 2 with 1 N HCl. After the sample was degassed, it was applied to a 1 × 25 cm column of Bio-Rad AG 50W-X4 (200–400 mesh) equilibrated with 0.05 N HCl. Elution with 0.05 N HCl gave the alkylated glutathione derivative centered at 267 mL; analysis as described below of an aliquot of the peak fraction on an amino acid analyzer showed the derivative to be of high purity and free of glutathione. It emerged from the analyzer at 10 min, compared to cysteic acid and carboxymethylcysteine as standards at 6 and 24 min, respectively. The precise concentration of the derivative in the peak fraction was determined by amino acid analyses (in triplicate) of the glycine and glutamic acid in total acid hydrolysates. These analyses, coupled with quantitation of the radioactivity in the same peak fraction, gave a specific radioactivity of 1.35 ± 0.07 cpm/pmol.

Amino Acid Analyses. Total acid hydrolysis of glutathione alkylated with bromoketoglutarate and reduced with sodium borohydride was achieved in evacuated (<50 μmHg) sealed tubes with 6 N HCl/0.1 M 2-mercaptoethanol at 110 °C for 21 h. Hydrolysates were dried on a Speed Vac concentrator (Savant Instruments Inc.) and subjected to chromatography on a Beckman 121M amino acid analyzer using Beckman's "3-h-single-column system".

Preparation of Solutions of 3-Bromo-2-ketoglutarate. A stock solution (110 mM) of BrKG (free acid, 216 g/mol) was prepared by dissolving a known weight in distilled water. This solution was stored at −80 °C for several months without detectable decomposition. The concentration of bromoketoglutaric acid was determined by weight and by its reaction with glutathione as described in the next paper (Bednar et al., 1982). The UV–vis absorption spectrum of bromoketoglutaric acid is not normally used for concentration determination since the extinction coefficient is not a linear function of concentration.[2]

[1] Abbreviations: BrKG, (*RS*)-3-bromo-2-ketoglutarate; 2-KG, 2-ketoglutarate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DEAE-cellulose, *O*-(diethylaminoethyl)cellulose; IC, isocitrate; DPN, diphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

[2] The absorbance of the stock solution (110 mM) at λ_{max} (341 nm) is 1.16 *A* units. The extinction coefficients at 341 nm were measured at the indicated concentrations in 0.1 M HOAc: 23.8 (5.0 mM), 26.9 (2.5 mM), and 29.0 M^{-1} cm^{-1} (1.25 mM).

Determination of the pK Values of BrKG. BrKG (1.02 mM) was titrated with NaOH (0.9639 N) using a Radiometer pH titrator. The titration was carried out in a stirred cell under nitrogen. No significant decomposition of BrKG was observed during these carefully done titrations.[3] The pKs were obtained from an iterative computer fit of the titration data (to minimize the least squares of the deviations) to a fourth power theoretical equation for the titration of a diprotic acid (Fleck, 1966). The average of four titrations yields $pK_1 = 2.30$ and $pK_2 = 4.28$.

Determination of the Intrinsic Reactivity of BrKG toward -SH Groups. BrKG (0.109 mM) and glutathione (0.112 mM) were incubated at pH 6.15 in 50 mM Mes buffer containing 2 mM $MnSO_4$ and 20% glycerol. At approximately 1 min intervals the concentration of thiol was determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid). An aliquot (500 μL) of the reaction mixture was added to 800 μL of potassium phosphate buffer (200 mM, pH 7.0) containing EDTA (20 mM) and Nbs_2 (1 mM). The increase in absorbance at 412 nm [13 600 M^{-1} cm^{-1} (Ellman, 1959)] of the thionitrophenolate ion was used to calculate the concentration of residual thiol. BrKG was shown not to react rapidly with thionitrophenolate ion under the assay conditions. The second-order rate constants (k) were obtained by fitting the data to

$$\ln [(B_0 - A_0)/A + 1] = \ln [B_0/A_0] - (A_0 - B_0)kt \quad (1)$$

where A_0 and B_0 are the initial concentrations of the two reactants (BrKG and GSH) and A is the concentration at time t of one of the reactants. The rate of the reaction of iodoacetate with glutathione was determined in a similar manner. Under the assumption that the unprotonated form of the thiol is the reactive species, the pH-independent second-order rate constant was calculated by using the equation

$$k_i = k(1 + 10^{pK_a - pH}) \quad (2)$$

Enzyme Preparation and Assay. Pig hearts were homogenized in a Waring blender as described by Ehrlich et al. (1981), and the DPN-dependent isocitrate dehydrogenase was precipitated with 60% ammonium sulfate. A differential ammonium sulfate fractionation was performed on the dissolved precipitate. The nominal percent saturation for the lower (~12.5%) and upper cuts (~20% at 4 °C) was determined by separate pilot precipitations on each batch of enzyme (E. V. Stevens and R. F. Colman, unpublished results). Dithiothreitol (0.1 mM) was added to all buffers used for dialysis and chromatography in order to increase the stability of the enzyme during the purification procedure (Ramachandran & Colman, 1978). The enzyme (2000 units) was further purified by ion-exchange chromatography on DEAE-cellulose (50 × 4.5 cm) (Shen et al., 1974). After chromatography on cellulose phosphate (Ramachandran & Colman, 1980), the enzyme exhibited specific activities around 25 enzyme units/mg. The purity of the enzyme was confirmed by electrophoresis in polyacrylamide gels containing 2% sodium dodecyl sulfate (Ramachandran & Colman, 1978). The band pattern upon isoelectric focusing in the presence of urea was the same as that reported by Ramachandran & Colman (1980).

Purified enzyme was dialyzed against 50 mM Mes buffer, pH 6.1, containing 2 mM $MnSO_4$ and 20% glycerol and stored in aliquots at −80 °C. Enzyme concentration was determined by multiplying the absorbance at 280 nm in a 1-cm light-path cuvette by 1.55 mg/mL ($E^{1\%}_{280} = 6.45$) (Shen et al., 1974). A value of 40 000 was used for the average subunit molecular weight (Ramachandran & Colman, 1980), since the enzyme is composed of three types of subunits of either 39 000 or 41 000 daltons.

Isocitrate dehydrogenase activity was determined at 25 °C in Tris–33 mM acetate buffer, pH 7.2, by measuring the increase in absorbance of DPNH at 340 mM (0.1 A unit full scale; 1-cm path length) using a Cary 219 spectrophotometer. A 5-μL aliquot of enzyme solution was added to 1.0 mL of a standard assay solution containing 20 mM isocitrate, 1 mM DPN, and 2 mM $MnSO_4$.

Kinetics of the Reaction of BrKG with DPN–Isocitrate Dehydrogenase. Isocitrate dehydrogenase (0.2 mg/mL) was incubated at 25 °C in 50 mM Mes, pH 6.1, containing 2 mM $MnSO_4$, 20% glycerol, and added ligands as indicated. Concentrated stock BrKG solution could not easily be titrated with NaOH without significant decomposition. For compensation for the contributed acidity of BrKG, buffers of pH up to 7.85 were prepared so that final concentrations of BrKG up to 10 mM could be used and a final pH of 6.1 maintained.[4] Two parallel incubations were always run: one with BrKG (experimental) and one without BrKG (control). Generally, the enzyme was preincubated at pH 6.6 for 90 min prior to addition of 110 mM BrKG (to yield a final concentration of 5 mM) to the experimental incubation and 0.22 N HCl to the control incubation to yield the same final pH. Aliquots (5 μL) were withdrawn at given time intervals and assayed by the standard assay. The activity of the experimental incubation solution (E) was divided by the activity of the control incubation (E_c) to correct for any small activity losses not due to BrKG. The rate constant for inactivation (k_{inact}) was obtained by a linear least-squares fit of the plot of ln (E/E_c) vs. time. The uncertainties at the 90% confidence limit were calculated by the method of Blaedel & Iverson (1976).

When loss of ADP activation was followed, aliquots (5 μL) of the incubation mixtures were assayed with and without ADP (2 mM) in an assay solution containing a low concentration of isocitrate (0.5 mM). Triethanolamine hydrochloride buffer (36 mM chloride, pH 7.1) containing 1 mM DPN was used for this assay. The rate constant for loss of ADP activation (k_{ADP}) was obtained by a linear least-squares fit of ln $[(R_t - R_\infty)/(R_0 - R_\infty)]$ vs. time, where R is the ratio of activity with and without ADP in the experimental incubation. R_0 is the ratio of activities at time zero (approximately 4.4), and R_∞ is the ratio at infinite time, which was taken as 1.0.

Determination of Covalent Incorporation of Radioactive BrKG. The stoichiometry of covalent incorporation was determined by utilizing a gel filtration column centrifugation technique (Fry et al., 1978; Penefsky, 1979; Tuszynski et al., 1980) to remove noncovalently bound reagent. A plastic syringe (5 mL) was filled with Sephadex G-50 (80 mesh) equilibrated with 50 mM Mes buffer (pH 6.1) containing 2 mM $MnSO_4$ and 20% glycerol. The syringe was centrifuged in a bench top clinical centrifuge (about 500 rpm) for 5.0 min before application of sample.

DPN–isocitrate dehydrogenase (0.2 mg/mL) was incubated with 8.3 mM [^{14}C]BrKG, and loss of activity was followed as described previously. At suitable time intervals, aliquots (200–400 μL) from both experimental and control incubations were withdrawn and applied dropwise to separate centrifuge

[3] The pK_a and end points were not significantly different at four different rates of titrant addition. Further, the number of equivalents of base added at the end point was in agreement with the concentration of BrKG. More equivalents would have been required if decomposition had occurred.

[4] Measurable decomposition occurs on mixing of BrKG (free acid) with 50 mM Mes buffer above pH 7.85. Further information on BrKG stability is given in the following paper (Bednar et al., 1982).

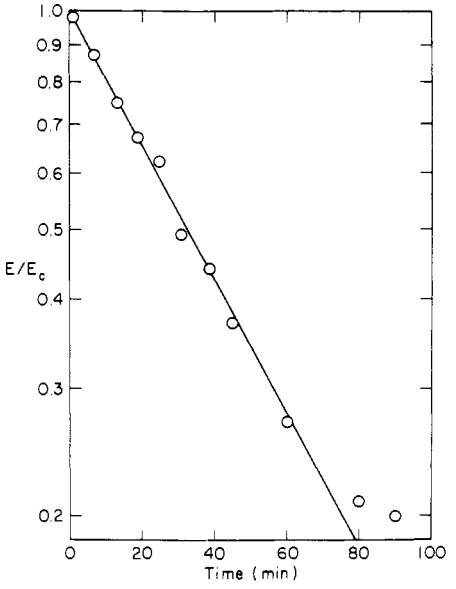

FIGURE 1: Time-dependent inactivation of DPN–isocitrate dehydrogenase by 3-bromo-2-ketoglutarate. Enzyme (0.2 mg/mL) was incubated with BrKG (8.4 mM) at pH 6 and 25 °C. The reaction in the experimental sample was initiated by addition of BrKG solution (free acid) to a solution of enzyme which had been preincubated at pH ~6.8 for 90 min. At zero time, a solution of HCl of the same normality as BrKG was added to the control sample. E and E_c are the enzymatic activities measured by the standard assay in the experimental and control incubations, respectively. The pseudo-first-order rate constant for inactivation is 0.0216 ± 0.0010 min^{-1} at the 90% confidence limit.

columns. Buffer (200 μL) was applied after the sample. The columns were centrifuged (about 500 rpm) for 5.0 min into a tared plastic centrifuge tube. In some cases, the effluent was passed through a second centrifuge column. The volume of effluent was determined by weight. The activity was measured by the standard assay. The protein concentration was measured by the Bio-Rad protein assay which is based on the Bradford method (1976). Unmodified DPN–isocitrate dehydrogenase was used as a standard. The concentration of covalently incorporated reagent was determined by counting an aliquot (50–200 μL) in 10 mL of ACS scintillation cocktail in a Packard Tri-Carb liquid scintillation counter. The correction factor for the quenching due to buffer was determined by making a standard addition of a known amount of counts.

Evaluation of the Catalytic Competence of Modified Enzyme. (*R*)-4-[4-^{3}H]DPNH was prepared from DPN (27 μmol) and [1-^{3}H]ethanol (1 mmol, 10^{10} dpm) using yeast alcohol dehydrogenase as the catalyst. The reaction goes essentially to completion when carried out in Tris base (1 M). The DPNH was isolated as the barium salt and converted to the sodium salt as described by Rafter & Colowick (1957).

DPN–isocitrate dehydrogenase was incubated with BrKG (experimental) and without BrKG (control). After 50–75% of the enzymatic activity was lost in the sample containing BrKG, the reactions were stopped by the gel filtration column centrifugation technique. Enzymes from both the experimental and control mixtures were next incubated for 18 h with 1 mM (*R*)-4-[4-^{3}H]DPNH. The samples were then brought to 6 M in urea, and the excess DPNH was removed by three successive centrifuge columns (equilibrated with 6 M urea). The protein concentration was determined by the Bio-Rad method, and the concentration of incorporated tritium was measured.

Results

Kinetics of Inactivation. Incubation of DPN-dependent isocitrate dehydrogenase with (*RS*)-3-bromo-2-ketoglutarate

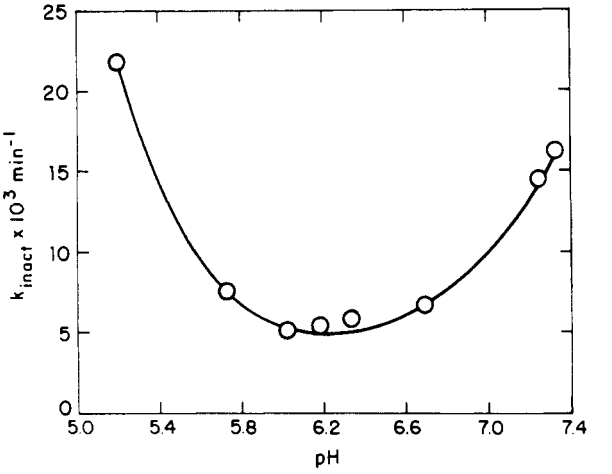

FIGURE 2: pH dependence of the inactivation of DPN–isocitrate dehydrogenase by 3-bromo-2-ketoglutarate. Enzyme (0.2 mg/mL) was incubated with BrKG (1 mM) in 50 mM buffer (Mes or Pipes) containing 2 mM $MnSO_4$ and 20% glycerol. The rate constant for loss of activity (k_{inact}) was measured as described under Experimental Procedures.

results in a rapid pseudo-first-order loss of enzyme activity (Figure 1). Activity is not restored by dialysis or gel filtration. Addition of glutathione (GSH) (10 mM) to the incubation mixture stops the inactivation, but no regain in activity is seen after 150 min at 0 °C.

The rate of inactivation as a function of pH shows a minimum at approximately pH 6.2 and increases at higher and lower pH values (Figure 2). The increased rate at higher pH may be attributable to a requirement for the unprotonated form of an enzymic amino acid. The data in the intermediate pH range (pH 6–6.7) could be fit to a pK of 6, but the data above pH 7 do not fit this pK. The increase at low pH may be due to a greater rate of reaction with monoprotonated BrKG (pK_2 = 4.28) than with the unprotonated form of the reagent. Alternatively, it is possible that different mechanisms of inactivation are operative at low, intermediate, and high pH values, i.e., that different enzymic groups react under these various conditions. The present studies were limited to the intermediate pH range (pH 6.1) with the aim of obtaining a limited reaction by a single mechanism.

A rate saturation effect is observed upon plotting the inactivation rate constant as a function of the reagent concentration. Figure 3 shows the double-reciprocal plot, which allows the calculation of K_{inact} (the reagent concentration yielding one-half the maximal rate of inactivation) for (*RS*)-BrKG of 6.2 mM. The k_{max} (pseudo-first-order rate constant at infinite reagent concentration) is 0.034 min^{-1}, which corresponds to a minimal half-time (T_{min}) of 20 min. This kinetic behavior is consistent with a model in which binding of the reagent (BrKG) to the enzyme (E) occurs prior to the irreversible formation of inactive enzyme (E–KG):

$$E + BrKG \underset{}{\overset{K_{inact}}{\rightleftharpoons}} E{\cdot}BrKG \xrightarrow{k_{max}} E\text{–}KG + Br^- \quad (3)$$

Intrinsic Reactivity of BrKG. The second-order rate constant for the reaction of BrKG with GSH is 67 M^{-1} at pH 6.15. If it is assumed that the unprotonated form of the thiol is the reactive species, and the pK_a of 8.66 approximates that of the –SH of glutathione (Stecker, 1968), eq 2 can be used to calculate 22 000 M^{-1} s^{-1} as the pH-independent rate constant for reaction with the BrKG dianion. Reaction of iodoacetate with GSH measured under similar conditions yields a constant of 0.0056 M^{-1} s^{-1} at pH 5.9 or, expressed as a pH-independent constant, 3.2 M^{-1} s^{-1}. This agrees well with a 3.8 M^{-1} s^{-1}

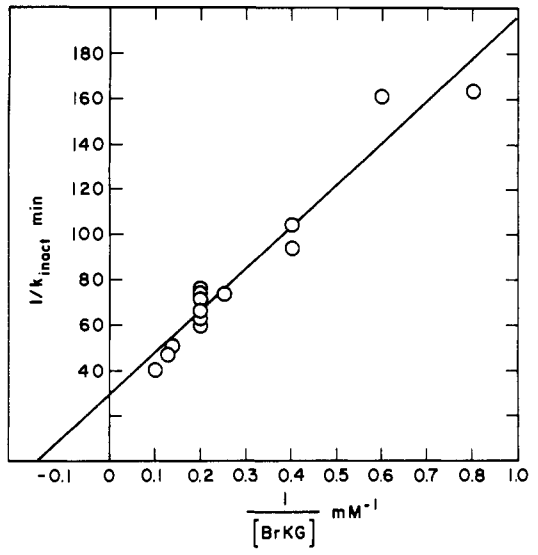

FIGURE 3: Dependence of the rate of inactivation on 3-bromo-2-ketoglutarate concentration. The rates of inactivation at different BrKG concentrations were calculated from a linear least-squares fit of E/E_c vs. time for t = 0–36 min. K_{inact} and k_{max} were calculated in accordance with the equation $1/k_{inact} = (K_{intact}/k_{max})(1/[BrKG]) + 1/k_{max}$.

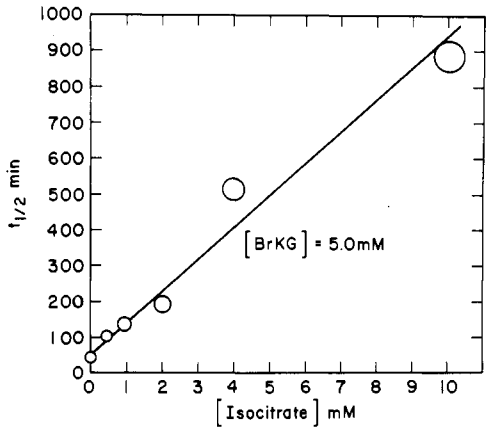

FIGURE 4: Isocitrate protection of DPN–isocitrate dehydrogenase from 3-bromo-2-ketoglutarate inactivation. The rate constants for inactivation at various concentrations of total DL-isocitrate were calculated from linear least-squares fits of ln E/E_c vs. time for t = 0–90 min. K_{IC} (the dissociation constant for isocitrate at the site at which it protects) was calculated from the model described by eq 3 and 4 using the equation $t_{1/2} = [T_{min}K_{intact}/([BrKG]K_{IC})][IC] + T_{min}(1 + K_{inact}/[BrKG])$.

constant, the average value calculated from data on the reactivity of iodoacetate with cysteine or GSH in the literature (Smythe, 1936; Dickens, 1933; Schroeder et al., 1933; Webb, 1966).

Effect of Added Ligands on the Rate of Inactivation of DPN–Isocitrate Dehydrogenase by BrKG. Isocitrate affords a high degree of protection of DPN–isocitrate dehydrogenase against inactivation by BrKG. A plot of the half-time of inactivation as a function of isocitrate concentration is shown in Figure 4. For a quantitative interpretation of this plot we used a model which combines the reversible binding of BrKG to the enzyme prior to inactivation (eq 3) with the reversible binding of isocitrate (IC) to the enzyme (eq 4). This model,

$$E + IC \overset{K_{IC}}{\rightleftharpoons} E{\cdot}IC \qquad (4)$$

which is described by the equation given in the legend of Figure 4, assumes that isocitrate and BrKG are linear competitive inhibitors. A dissociation constant of 280 μM was calculated

Table I: Effect of Ligands on Isocitrate (IC) Protection[a]

additions to reaction mixture	$k_{inact} \times 10^3$ (min^{-1})	K_{IC} (mM)
(1) sodium acetate (0.2 M)	13.2 ± 1.0	1.9
(2) sodium acetate (0.2 M) + IC (2 mM)	6.4 ± 0.8	
(3) sodium sulfate (0.1 M)	10.2 ± 0.7	2.6
(4) sodium sulfate (0.1 M) + IC (2 mM)	5.8 ± 0.9	
(5) glutarate (0.1 M)	11.6 ± 1.0	1.8
(6) glutarate (0.1 M) + IC (2 mM)	5.5 ± 0.8	
(7) 2-ketoglutarate (0.1 M)	12.3 ± 1.0	
(8) 2-ketoglutarate (0.1 M) + IC (2 mM)	8.1 ± 0.9	

[a] DPN–isocitrate dehydrogenase was incubated with BrKG (5 mM), $MnSO_4$ (2 mM), and the additional compounds indicated. K_{IC} is defined by the equation $k(+IC)/k(-IC) = 1/(1 + [IC]/K_{IC})$.

for total DL-isocitrate from the site at which it protects. This value is in good agreement with a directly determined binding constant of 320 μM for isocitrate at the active site (Ehrlich & Colman, 1981). The addition of metal ion has little effect on the inactivation rate: at 5 mM BrKG, k_{inact} = 0.0151 ± 0.0006 min^{-1} in the presence of 2 mM added $MnSO_4$, whereas k_{inact} = 0.0148 ± 0.0014 min^{-1} when 1 mM EDTA was added to chelate the <0.4 mM $MnSO_4$ contributed by the dialyzed enzyme. In contrast, metal is essential for effective protection by isocitrate: k_{inact} = 0.0023 ± 0.0007 min^{-1} in the presence of 2 mM isocitrate plus 2 mM $MnSO_4$, but its value is as much as 0.0122 ± 0.0018 min^{-1} when 2 mM isocitrate is added together with 1 mM EDTA. This result is consistent with the proposition that manganous ion is required for the productive binding of isocitrate (Cohen & Colman, 1972, 1974). Thus, isocitrate protects by a specific interaction with the active site.

2-Ketoglutarate, the product of the enzyme-catalyzed reaction with isocitrate, does not offer significant protection even at 0.1 M in the presence of 3 mM $MnSO_4$ (k_{inact} = 0.0123 ± 0.001 min^{-1}). Although a K_M of 11 mM has been measured by Shen & Colman (1975) for the reductive carboxylation of 2-ketoglutarate, there is no evidence that 2-ketoglutarate binds to free enzyme. The experiments testing the effect of ligands on isocitrate protection (Table I) were performed in order to determine if 2-ketoglutarate was binding at the active site with a dissociation constant of 11 mM and not offering protection against inactivation. Since the K_M for isocitrate is increased in the presence of high salt (Hayman & Colman, 1977), values of K_{IC} (the concentration of isocitrate yielding 50% protection) were determined in the presence of 0.1 M sodium sulfate, 0.2 M sodium acetate, and 0.1 M glutarate as well as 0.1 M 2-ketoglutarate to evaluate and compensate for the effect of addition of 0.1 M 2-ketoglutarate which is attributable to increased ionic strength.

A model in which 2-ketoglutarate binds at the active site and displaces isocitrate but does not itself offer protection is described:[5]

$$E + BrKG \xrightarrow{k_{app}} E\text{–}KG + Br^-$$

$$E + 2\text{-}KG \overset{K_{2\text{-}KG}}{\rightleftharpoons} E{\cdot}2\text{-}KG$$

$$E{\cdot}2\text{-}KG + BrKG \xrightarrow{k_{app}} E\text{–}KG{\cdot}2\text{-}KG + Br^-$$

$$E + IC \overset{K_{IC}}{\rightleftharpoons} E{\cdot}IC \qquad (5)$$

This model predicts that the addition of 2-ketoglutarate to-

[5] The prior binding of BrKG was omitted from this model since the concentration of BrKG was not varied in the experiments in Table II. The apparent rate constant is a composite of K_{inact} and k_{max} for BrKG under conditions of high salt.

Table II: Effect of Nucleotides on the Inactivation Rate by BrKG[a]

additions to reaction mixture	$k_{inact} \times 10^3$ (min^{-1})	K_i (μM)
(1) $MnSO_4$ (2 mM)	15.1 ± 0.6	
(2) DPN (0.53 mM) + $MnSO_4$ (2 mM)	11.3 ± 0.9	810
(3) DPN (2.0 mM) + $MnSO_4$ (2 mM)	6.0 ± 0.8	730
(4) DPN (5.0 mM) + $MnSO_4$ (2 mM)	3.8 ± 0.7	930
(5) ADP (0.47 mM) + $MnSO_4$ (2 mM)	3.9 ± 0.7	1.9[b]
(6) ADP (0.47 mM) + EDTA (1 mM)	12.2 ± 0.7	
(7) ADP (5.4 mM) + $MnSO_4$ (2 mM)	1.1 ± 1.4	4.8[b]

[a] DPN-isocitrate dehydrogenase was incubated with BrKG (5 mM) and the indicated additional ligands. K_i is defined by the equation $k(+\text{Nuc})/k(-\text{Nuc}) = (1 + [\text{BrKG}]/K_{inact})/(1 + [\text{BrKG}]/K_{inact} + [\text{Nuc}]/K_i)$. [b] For ADP^{3-}.

gether with isocitrate would weaken the protection provided by the same concentration of isocitrate alone. The model allows us to calculate the ratio of rate constants for inactivation in the presence and absence of isocitrate [k(+2-KG, +IC)/k(+2-KG, −IC)]. This ratio is defined by

$$\frac{k(+2\text{-KG}, +\text{IC})}{k(+2\text{-KG}, -\text{IC})} = \frac{1 + [2\text{-KG}]/K_{2\text{-KG}}}{1 + [2\text{-KG}]/K_{2\text{-KG}} + [\text{IC}]/K_{\text{IC}}} \quad (6)$$

If we use the average K_{IC} (2.1 mM) obtained from Table I, lines 1–6, and assume that the 2-ketoglutarate binds at the active site with a K_D equal to its K_M, then the expected rate constant for inactivation in the presence of 2 mM isocitrate and 0.1 M 2-ketoglutarate would be 0.0112 min^{-1}. The observed rate constant (Table I, line 8) is only 0.0081 min^{-1}. This discrepancy shows that 2-ketoglutarate cannot have a K_D of 11 mM and still be competitive with isocitrate. If we calculate a $K_{2\text{-KG}}$ which fits the experimental data, a constant of 150 mM is obtained. Clearly, if 2-ketoglutarate is competitive with isocitrate, its binding must be very weak. The lack of significant protection by 2-ketoglutarate against inactivation is likely to be due to very poor binding of 2-ketoglutarate to free enzyme.

The weak protection offered by the coenzyme DPN against inactivation by BrKG is illustrated by the data in Table II. A K_{DPN} of about 820 μM can be calculated if we assume that DPN and BrKG are linear competitive inhibitors. However, the K_D for DPN binding at the coenzyme site has been measured as 55 μM (Ehrlich & Colman, 1981). The observed protection is thus unlikely to be caused by a specific interaction with the coenzyme site.

The activator ADP offers significant protection to an extent which is reasonably consistent with its directly determined binding constant. Under the assumption that ADP and BrKG are competitive, an average binding constant for ADP ($K_{ADP^{3-}}$) of 3.3 μM can be calculated from the data in Table II (lines 5 and 7). This can be compared to a 2.6 μM binding constant calculated from the data of Ehrlich & Colman (1981). It can be seen (Table II, lines 5 and 6) that metal is essential for effective protection by ADP, in agreement with the observed metal requirement for the directly measured tight binding of ADP (Ehrlich & Colman, 1981).

Since ADP protects against loss of catalytic activity, the effect of the BrKG reaction on the allosteric properties of DPN-isocitrate dehydrogenase was evaluated. This enzyme is allosterically activated by ADP which lowers the K_M for isocitrate but does not affect the maximum velocity (Cohen & Colman, 1972). The effect of ADP is conveniently observed as an increase in the initial velocity caused when the nucleotide is added to solutions containing nonsaturating concentrations of isocitrate (Hayman & Colman, 1978). The reaction of the

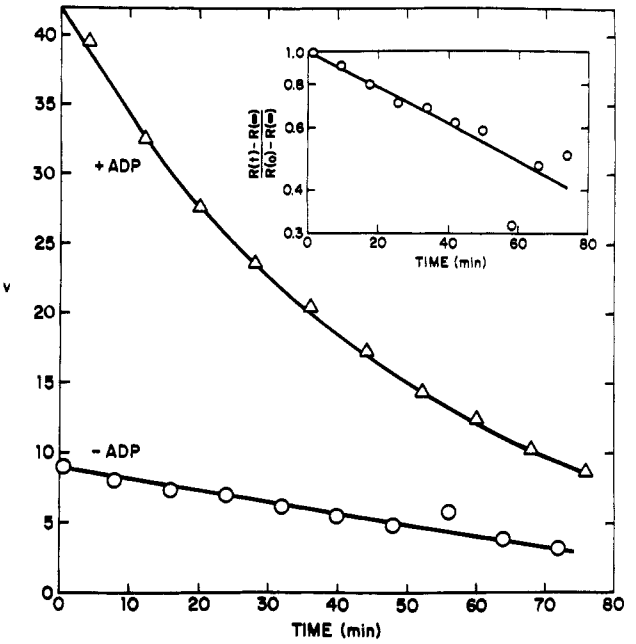

FIGURE 5: Time-dependent loss of ADP activation upon treatment with 3-bromo-2-ketoglutarate. DPN-isocitrate dehydrogenase was incubated with BrKG (5 mM) as described in Figure 1. At the indicated times, aliquots were removed and assayed for enzyme activity at a low level of isocitrate (0.5 mM) either in the presence of 2 mM ADP (△) or in the absence of ADP (○). The inset is a semilog plot of loss of ADP activation. $R(t)$ is a measure of the ADP activation, i.e., the ratio of activity with ADP to the activity without ADP. The y axis is a measure of the fractional residual ADP activation. $R(0)$ is 4.4 and $R(\infty)$ is taken as 1.0. The rate constant for loss of ADP activation is 0.0123 ± 0.0044 min^{-1}, and the rate of loss of activity in the assay without ADP (calculated as shown in Figure 1) is 0.0131 ± 0.0030 min^{-1}.

enzyme and BrKG was followed by assaying enzyme activity in the presence and absence of ADP. Figure 5 shows that there is a time-dependent loss of ADP activation. If the end point is assumed to be complete loss of ADP activation, then the rate of loss of ADP activation can be calculated as shown in the inset of Figure 5. The rate constant (k_{ADP} = 0.0123 ± 0.0044 min^{-1}) for loss of ADP activation is the same as the rate constant (k_{inact} = 0.0131 ± 0.0030 min^{-1}) for loss of activity. The equality of the rate constants suggests that both effects are the result of the same molecular event. Further evidence for a single molecular event is seen from the equivalent protection against loss of ADP activation and loss of activity by 0.4 mM isocitrate and 2 mM $MnSO_4$ (k_{ADP} = 0.0046 ± 0.0012 min^{-1} and k_{inact} = 0.0037 ± 0.0008 min^{-1}). In addition, 0.3 mM ADP plus 2 mM $MnSO_4$ decreases both rates equally (k_{ADP} = 0.0026 ± 0.0012 min^{-1} and k_{inact} = 0.0024 ± 0.0013 min^{-1}). The measured loss of ADP activation must result from a change in the influence of ADP on the catalytic activity of unmodified subunits. Therefore, the effect of BrKG on the ADP activation presumably results from interaction between modified and unmodified subunits.

Stoichiometry of Covalent Incorporation of Reagent into Enzyme. The moles of covalently bound reagent were determined by the centrifuge gel filtration technique described under Experimental Procedures. The recovery of protein for the experimental incubation solution was sometimes lower than that of the control incubation solution, especially for enzyme of less than 25% residual activity. However, in all cases the specific activities before and after the column were not significantly different. This observation suggests that there was no appreciable selection of native or modified enzyme during gel filtration. Passing the protein through two successive

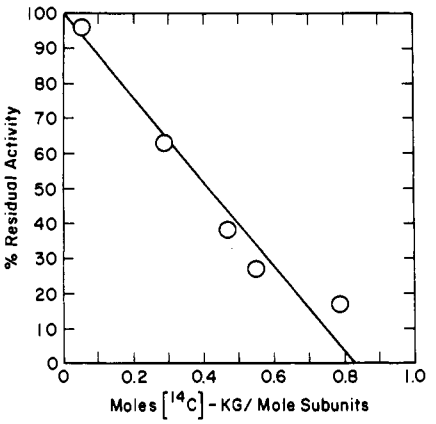

FIGURE 6: Residual activity as a function of incorporation for the inactivation of DPN–isocitrate dehydrogenase by 3-bromo-2-ketoglutarate. Isocitrate dehydrogenase was incubated with [^{14}C]BrKG (8.3 mM) as described in Figure 1. The amount of covalent incorporation was determined after centrifugal gel filtration. The percent residual activity is defined by $E/E_c \times 100$.

columns did not appear to change significantly the measured incorporation.

The correlation between loss of activity and the moles of covalently bound reagent per average subunit is shown in Figure 6. Extrapolation to 0% residual activity yields a value of 0.83 mol of [^{14}C]KG per enzyme subunit. This plot suggests that, on the average, modification of no more than one amino acid residue per average subunit is responsible for the loss in enzyme activity.

When DPN–isocitrate dehydrogenase was incubated with [^{14}C]BrKG (8.8 mM) in the presence of 10 mM isocitrate and 2 mM $MnSO_4$ for 100 min, less than 19% of the activity was lost but 0.37 mol of [^{14}C]KG was incorporated per subunit. If isocitrate were not present in the incubation solution, 0.80 mol of [^{14}C]KG/subunit would have been incorporated over 100 min. This decrease of 0.43 mol of [^{14}C]KG/subunit in the presence of isocitrate indicates that this substrate significantly slows the rate of modification. However, if we compare the incorporation for loss of 19% activity in the presence of isocitrate with the results of Figure 6, we see an extra 0.20 mol of [^{14}C]KG/subunit for 19% inactivation in the presence of isocitrate. The relatively greater incorporation of radioactivity in the presence of isocitrate may be due to a slow nonspecific modification which occurs during the 90-min longer incubation period required to cause 19% reduction in activity in the presence of isocitrate. It is also possible that isocitrate might promote reaction with groups not normally modified in the absence of isocitrate.

Incubation of [^{14}C]BrKG (8.3 mM) with DPN–isocitrate dehydrogenase in 5 M urea results in the incorporation of 3.7 mol of [^{14}C]KG/subunit within 5 min and 5.0 mol of [^{14}C]-KG/subunit after 90 min. In the native state only about 0.8 mol of [^{14}C]KG/subunit would be incorporated at 90 min, indicating that the native tertiary structure is essential in limiting the reaction of BrKG with the enzyme.

BrKG as a Substrate. As judged by the oxidation of DPNH, 50% of (*RS*)-3-bromo-2-ketoglutarate is a substrate for DPN–isocitrate dehydrogenase. DPNH oxidation with BrKG as a cosubstrate does not require carbon dioxide nor is there any release of bromide ion.[6] The product is presumed

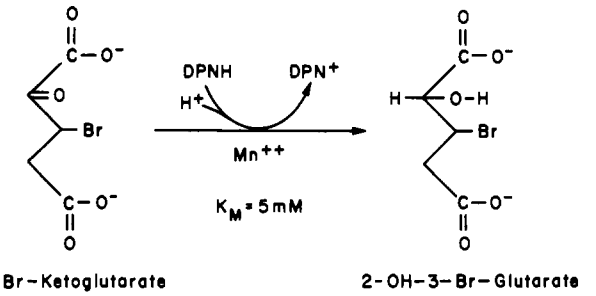

FIGURE 7: Reaction of 3-bromo-2-ketoglutarate as a substrate for DPN–isocitrate dehydrogenase.

to be 2-hydroxy-3-bromoglutarate (Figure 7). The addition of EDTA causes a loss of all detectable turnover of BrKG, indicating that the catalytic reaction has an absolute requirement for divalent metal ion.

Under the same conditions (Experimental Procedures) used for the inactivation reaction (except for the addition of 2 mM DPNH), a V_{max} of 8 units/mg (k_{cat} = 320 min^{-1}) and a K_M for BrKG of 5.2 mM were measured by using the time-dependent Henri–Michaelis–Menten equation (Segel, 1975). The measured V_{max} is very similar to the V_{max} for the oxidative decarboxylation of isocitrate,[7] illustrating the efficiency of the enzyme in catalyzing this reaction. Catalytic turnover is very fast compared to inactivation. Approximately 11 000 molecules of BrKG would be expected to turn over for each inactivation. Thus, one would not expect to detect inactivation during the monitoring of BrKG as a substrate. The K_M compares very well with the K_{inact} of 6.2 mM, suggesting that the function of BrKG as a substrate and as an inactivator may involve binding to the same site.[8]

The ability of the enzyme to catalyze the reduction of BrKG without the carboxylation led us to look closely at the possibility of an enzyme-catalyzed reduction of 2-ketoglutarate. In the absence of CO_2, the enzyme can catalyze the reduction of 2-ketoglutarate and oxidation of DPNH. The formation of DPN was confirmed by addition of isocitrate which converted the DPN back to DPNH. The addition of EDTA

[6] Only 7–8% of the Br^- expected for a stoichiometric release was detected with an Orion bromide electrode. This slight loss of Br^- is probably due to buffer-catalyzed decomposition of BrKG (Bednar et al., 1982).

[7] The enzyme used had a specific activity of about 20 units/mg in a standard assay. When the data of Ramachandran & Colman (1977) are used, a V_{max} of about 7 units/mg would be expected for oxidative decarboxylation of isocitrate at pH 6.1.

[8] It should be pointed out that, alternatively, the agreement of K_M and K_{inact} could be explained by a different model: (1) One isomer (the substrate, S) binds at the active site but does not inactivate the enzyme due to the absence of a suitably positioned nucleophilic group in the active site. (2) The other isomer (the inactivator, R) either binds at the active site with a weaker affinity or may react by a bimolecular process. This model implies that the binding of the "substrate isomer" at the active site, either directly or indirectly, slows the rate of inactivation by the other isomer:

$$\begin{aligned} &E + R \underset{}{\overset{K_R}{\rightleftharpoons}} E{\cdot}R \xrightarrow{k_1} E\text{-}R \\ &+ \qquad\qquad (7) \\ &S \overset{K_S}{\rightleftharpoons} E{\cdot}S \\ &[R] = [S] \end{aligned}$$

This model is described by the equation

$$K_{inact} = \frac{[K_S/(K_R + K_S)]k_1[R]}{K_{inact} + [R]} \qquad (8)$$

where $K_{inact} = K_S[K_R/(K_R + K_S)]$. In this model, the observed K_{inact} will reflect the binding of the substrate isomer (S) if $K_R \gg K_S$.

completely prevented the reaction, indicating an absolute requirement for metal. Under the conditions described above for determination of the K_M for BrKG (except that [isocitrate dehydrogenase] = 1 mg/mL), a V_{max} of 0.017 unit/mg (k_{cat} = 0.68 min^{-1}) and a K_M for 2-ketoglutarate of 11 mM were determined. The K_M is essentially the same as that seen by Shen & Colman (1975) for the reductive carboxylation of 2-ketoglutarate at the same pH. Since the V_{max} is only about 3% of that reported by Shen & Colman (1975) for the reductive carboxylation, it is not surprising that this reaction was not previously detected. The 1000-fold greater V_{max}/K_M for BrKG compared to 2-ketoglutarate may suggest the presence of a significant binding energy between Br and the enzyme, although it should also be considered that the electron-withdrawing ability of bromine may contribute part of the enhanced rate of reduction of BrKG.

Evaluation of the Catalytic Competence of Modified Enzyme. Tritium-labeled reduced coenzyme was synthesized in order to test whether the covalently modified enzyme was catalytically competent. If modification occurs at the active site and the modified enzyme is catalytically competent, then a single turnover transfer of tritium from the coenzyme to covalently bound reagent should be possible. The net result of this transfer would be to covalently incorporate tritium into the protein. Attempts to measure tritium incorporation yielded only a low level (<0.002 mol/subunit) of tritium incorporation into enzyme from both experimental and control incubations. The BrKG-modified enzyme appears not to be catalytically competent.

Discussion

Affinity labels frequently are designed to incorporate an electrophilic group which can react with nucleophilic amino acid side chains of a protein to form a stable covalent linkage. By far the most reactive amino acid side chain is the thiol of cysteine. The reactivity toward –SH offers a covenient measure of the reactivity of BrKG. Like other α-halo ketones,[9] BrKG is a very reactive molecule. It is almost 7000 times more reactive than the commonly used electrophile iodoacetate and is comparable in reactivity to the reagent 5,5′-dithiobis(2-nitrobenzoate).[10]

BrKG inactivates DPN–isocitrate dehydrogenase with a second-order rate constant (k_{max}/K_{inact} = 0.091 M^{-1} s^{-1}) which is only 0.1% as great as that of the reaction of BrKG with GSH at pH 6.15. For such a reactive reagent to be specific, it is necessary that the enzyme not have many –SH groups which are readily accessible to the reagent; however, the accessibility of thiols to a particular reagent is not always easy to predict. BrKG inactivates DPN–isocitrate dehydrogenase about 13 times as fast as does iodoacetate (k = 0.0072 M^{-1} s^{-1} at the same pH) (Mauck & Colman, 1976), but is much more specific. With 16% residual activity, 2.8 mol of [^{14}C]-acetate/subunit is incorporated as a result of reaction with iodoacetate (Mauck & Colman, 1976). In contrast, BrKG causes incorporation of only 0.80 mol of [^{14}C]KG/subunit when 17% residual activity remains. Even though BrKG is more reactive than iodoacetate, it modified fewer groups on this enzyme. The native tertiary structure of isocitrate dehydrogenase must be responsible for limiting the reaction of BrKG, since when the enzyme is dissolved in 5 M urea, as many as 5.0 mol of [^{14}C]KG/subunit can be incorporated. In order to function as an effective specific affinity label, a reagent which contains a highly reactive electrophilic group not only must have an enhanced affinity for the active site but also must exhibit decreased access toward reactive groups on the enzyme surface.

Several lines of evidence support the notion that 3-bromo-2-ketoglutarate acts as an affinity label for the active site of DPN–isocitrate dehydrogenase. BrKG shows a close structural analogy to the natural substrates of this enzyme. In fact, in the presence of reduced coenzyme, bromoketoglutarate is a substrate. The electron-rich bromine atom may resemble the carboxylate of isocitrate or of the enzyme-bound intermediate oxalosuccinate so that BrKG can occupy the normal site for these substrates. The rate saturation behavior observed from the dependence of inactivation rate constant on BrKG concentration accords with the expectation for an affinity label. The good agreement of K_M with the K_{inact} suggests that BrKG as a substrate and as an inactivator may bind at the same site. The dependence of inactivation on the moles of reagent incorporated indicates that modification of no more than one residue per subunit is responsible for complete loss of activity, suggesting an extremely limited extent of reaction. The specific protection by the substrate isocitrate fulfills a very important criterion for an active-site modification. The lack of specific protection by the product 2-ketoglutarate, while initially puzzling, can probably be ascribed to the poor binding of 2-ketoglutarate to free enzyme. The higher affinity of BrKG (as compared to 2-ketoglutarate) for the free enzyme must result from a positive interaction of the bromine atom with the enzyme.

Groman et al. (1975) have proposed catalytic competence as a new criterion of active site labeling, catalytic competence being the ability of an enzyme to catalyze its normal reaction with a covalently bound substrate. Since the BrKG-modified enzyme contains a ketone bound at the active site, it was thought possible that the enzyme might catalyze a single turnover conversion of the ketone to alcohol. However, using tritium-labeled coenzyme, we found no tritium incorporated into the enzyme that could be attributed to the conversion of ketone into alcohol. While the observation of catalytic competence can provide direct evidence for an active-site modification, the lack of catalytic competence does not exclude such active-site modification, since the formation of a covalent linkage may prevent the bound substrate from achieving the orientation required for enzymic catalysis. It is possible that in this case the lack of catalytic competence should have been anticipated. If the modified residue were to act as a general acid to protonate the carbonyl oxygen in the formation of the alcohol, its modification would render the enzyme catalytically incompetent.

The loss of the allosteric ADP activation upon modification and the specific protection provided by ADP against inactivation might not have been expected for an active-site modification. However, there is significant evidence of interaction

[9] A convenient measure of intrinsic reactivity of a chemical-modifying reagent is the pH-independent rate constant for the reaction of an electrophilic moiety with the thiol of GSH or *N*-AcCys. On the basis of the data of Hartman (1970), the pH-independent rate constants for the reaction of the α-halo ketones 3-chloroacetol phosphate, 3-bromoacetol phosphate, and 3-iodoacetol phosphate (oxidation of GSH for the iodo compound) with GSH are 10.3, 27 400, and 416 000 M^{-1} s^{-1}, respectively. The commonly used haloacetyl chemical modifying reagents are less reactive; the rate constants for bromoacetate and iodoacetate with GSH are 2.7 and 3.8 M^{-1} s^{-1}, respectively (Smythe, 1936; Dickens, 1933; Schroeder et al., 1933; Webb, 1966). Bromoethyl-AMP derivatives have been shown to react with thiols with a pH-independent rate constant of 3×10^{-4} M^{-1} s^{-1} (Bednar, 1982). α-Halo ketones are much more reactive than alkyl halides.

[10] The constant for the reaction of Nbs_2 with GSH has been reported as 149 M^{-1} s^{-1} at pH 6.5 (Degani & Patchornik, 1974). From this value, a pH-independent rate constant of 21 700 M^{-1} s^{-1} may be calculated.

between the ADP site and the isocitrate site of this enzyme. ADP enhances the affinity of isocitrate for the enzyme as seen kinetically by the allosteric ADP activation (Cohen & Colman, 1972) and also by direct binding experiments (Ehrlich & Colman, 1981). ADP also protects against modification of the isocitrate site by 2,3-butanedione (Hayman & Colman, 1978). The equivalence of the rate constants for loss of ADP activation and for loss of activity upon reaction with BrKG suggests that both processes may result from the same molecular event. The structural similarity between BrKG and the natural substrate, the ability of BrKG to interact with the active site as demonstrated by its behavior as a substrate, and the agreement of K_M and K_{inact} make the active site the most probable site modified by this reagent.

If BrKG indeed reacts at the active site of DPN-dependent isocitrate dehydrogenase, it is apparent that any subunit which is modified is inactive. The observation of ADP activation in partially modified enzyme must therefore be a measure of the effect of ADP on the activity of unmodified subunits. If this were an enzyme with totally independent subunits, one would have expected the catalytic and regulatory properties of partially active enzyme to be the same as those of native enzyme. On the contrary, since a time-dependent loss of the ADP activation is observed, it is apparent that there must be communication between the modified and unmodified subunits and that inactivation of a subunit causes a decrease in the effect of ADP on the activity of other subunits.

DPN–isocitrate dehydrogenase is composed of structurally dissimilar subunits in the ratio 2:1:1 (Ramachandran & Colman, 1980). Since each of the subunits has a molecular weight of 39 000 or 41 000, the minimum size of an intact enzyme should consist of found subunits and have a molecular weight of 160 000. The correlation between loss of activity and amount of covalent incorporation suggests that, on average, modification of three to four of the subunits is required to completely inactivate the enzyme. This result may suggest that, despite the structural dissimilarity, every subunit potentially has an active site.

Evidence presented in the following paper (Bednar et al., 1982) indicates that cysteine is the site of modification by BrKG. It is possible that a cysteine residue in the active site of the pig heart DPN-dependent isocitrate dehydrogenase serves as a general base in the catalytic mechanism of this enzyme.

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