3,4-Didehydro-2-ketoglutarate: An Affinity Label for Diphosphopyridine Nucleotide Dependent Isocitrate Dehydrogenase†

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ABSTRACT: The 2-ketoglutarate analogue 3-bromo-2-ketoglutarate decomposes in buffered solution to produce a compound which can react with nucleophiles. At pH 6.15 in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) buffer containing 2 mM $MnSO_4$ and 20% glycerol, both the loss of bromide and the loss of the ability to serve as a substrate for pig heart TPN-dependent isocitrate dehydrogenase occur with a half-time of 1.6 h; however, the loss of the ability to react with glutathione occurs with a half-time of 7 h. Presumably, elimination of HBr yields an unsaturated compound, 3,4-didehydro-2-ketoglutarate, which would be expected to add nucleophiles by a Michael-type addition at C-4. This decomposition product progressively inactivates pig heart DPN-dependent isocitrate dehydrogenase. With increasing concentrations of the reagent, the reaction exhibits a rate saturation: the minimum inactivation half-time is 35 min with a K_{inact} of 1.5 mM. Specific protection against inactivation is afforded by the substrate isocitrate, while the coenzymes DPN and DPNH yield only weak protection. Incubation of the presumed 3,4-didehydro-2-ketoglutarate with the enzyme results in a loss of allosteric ADP activation at the same rate as inactivation. Both isocitrate and ADP afford equal protection against loss of activity and loss of ADP activation, implying that both processes may result from the same molecular event. Since the loss of ADP activation measures the effect of the allosteric compound on unmodified catalytically active subunits, there is strong evidence of interaction between modified and unmodified subunits. Complete loss of enzymatic activity correlates with the covalent incorporation of ~1.0 mol of reagent/mol of average subunit. These results suggest that 3,4-didehydro-2-ketoglutarate functions as an affinity label of the substrate binding site of DPN-dependent isocitrate dehydrogenase. The stoichiometry of incorporation suggests that each of the structurally distinct subunits of the enzyme must be modified for complete loss of activity. A cysteine residue was identified as the prime candidate for the amino acid modified by both 3-bromo-2-ketoglutarate and the putative 3,4-didehydro-2-ketoglutarate. This cysteine residue may function as a general base in the enzymic reaction mechanism.

2-Ketoglutarate is a 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) (Hartman, 1981). In light of the large number of enzymes which bind 2-ketoglutarate, 3-bromo-2-ketoglutarate was proposed as a potential chemical probe of 2-ketoglutarate binding sites (Hartman, 1981). In the previous paper (Bednar et al., 1982), 3-bromo-2-ketoglutarate, a substrate analogue of 2-ketoglutarate, was shown to be a substrate and an inactivator of DPN-dependent isocitrate dehydrogenase. In this paper the stability of 3-bromo-2-ketoglutarate is evaluated. 3-Bromo-2-ketoglutarate is shown to decompose into a compound, presumed to be 3,4-didehydro-2-ketoglutarate, which can react with nucleophiles such as cysteine or glutathione. Evidence is presented indicating that 3,4-didehydro-2-ketoglutarate (eneKG)[1] inactivates DPN-dependent isocitrate dehydrogenase [*threo*-D_s-isocitrate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.41], exhibiting many of the characteristics expected of an affinity label. The decomposition product of 3-bromo-2-ketoglutarate is proposed as another potential affinity label of 2-ketoglutarate binding sites. A preliminary version of this work has been presented (Bednar et al., 1981).

Experimental Procedures

Materials. Bovine liver glutamate dehydrogenase and TPN–isocitrate dehydrogenase were purchased from Boehringer Mannheim Corp. DPN–isocitrate dehydrogenase, (*RS*)-3-bromo-2-ketoglutaric acid (BrKG), and other materials were prepared or purchased as described previously (Bednar et al., 1982).

Decomposition of BrKG. The decomposition of BrKG was studied at 25 °C in 50 mM Mes buffer (pH 6.1) containing 2 mM $MnSO_4$ and 20% glycerol (buffer A). A stock solution (110 mM) of BrKG (free acid) was prepared by weight. For compensation for the acidity of BrKG, the incubations were started by adding the BrKG (5 mM final concentration) to a higher pH buffer (pH 6.6) in order to attain a final pH of 6.1. The incubations were generally followed for a 24-h period. Several parameters were analyzed over this time period: formation of bromide ion, loss of reactivity with glutathione, loss of activity as a substrate for TPN–isocitrate dehydrogenase, appearance of a substrate for TPN–isocitrate dehydrogenase, and appearance of a substrate for glutamate dehydrogenase. The details of these assays are given below. The amount of decarboxylation of uniformly labeled [^{14}C]-BrKG was determined after 22 h by trapping $^{14}CO_2$ in 10% (w/w) NaOH.

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[1] Abbreviations: BrKG, (*RS*)-3-bromo-2-ketoglutarate; eneKG, 3,4-didehydro-2-ketoglutarate (3-ene-2-ketoglutarate in figures); 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); IC, isocitrate; Taps, 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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The decomposition of BrKG was also studied in the absence of buffer by using a Radiometer pH stat. The production of protons caused by the decomposition of BrKG (1 mM) was followed in a stirred cell under nitrogen at pH 8.0 and 10.0. The resulting half-times were 2 h and 6 min, respectively.

Assay for Compounds Capable of Reaction with Glutathione. The concentrations of highly reactive electrophilic compounds were measured by their reaction with glutathione (GSH). The general procedure was to allow an aliquot (0.6–0.8 mM) of the sample to react with GSH (1 mM) in 100 mM Taps buffer, pH 7.4, until the reaction was complete (5 min). The residual –SH concentration was determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) to produce thionitrobenzoate as indicated by the increase in the A_{412nm} [13 600 M^{-1} cm^{-1} (Ellman, 1959)]. An aliquot of the glutathione reaction mixture was added to Nbs_2 (1 mM) in 100 mM potassium phosphate buffer (pH 7.0) and 10 mM EDTA. The concentration of the compound which had reacted with glutathione equals the difference between 1 mM and the measured –SH concentration in the glutathione reaction mixture.

Assay for the Formation of Bromide Ion. The formation of bromide ion resulting from the decomposition of BrKG was followed by using an Orion bromide ion specific electrode. Standard bromide solutions (0.25–5.0 mM) were prepared in the same buffer as the sample. All measurements were taken at 25 °C with frequent restandardization of the electrode using fresh standard solutions.

Assay for Substrate(s) of TPN–Isocitrate Dehydrogenase. An aliquot (containing 50–80 μmol) of the sample was added to 1.0 mL of assay solution containing 150 μM TPNH, 2 mM $MnSO_4$, Tris (33 mM acetate), pH 7.2, and 0.25 unit of TPN–isocitrate dehydrogenase. The concentration of substrate was calculated from the decrease in absorbance at 340 nm (6.22 mM^{-1} cm^{-1}) followed with a Cary 219 spectrophotometer (1 *A* unit full scale). The reaction goes to an end point within a few minutes. One isomer of (*RS*)-3-BrKG is a substrate for TPN–isocitrate dehydrogenase (Hartman, 1981).

Assay for Substrate(s) of Glutamate Dehydrogenase. An aliquot of sample was added to 1.0 mL of assay solution containing 220 μM TPNH, 67 mM potassium phosphate (pH 7.6), 50 mM ammonium chloride, and 0.24 mg of bovine liver glutamate dehydrogenase. The decrease in TPNH absorbance (340 nm) was followed as a function of time. There was an initial rapid decrease in A_{340nm}, and after several minutes only a slow, essentially constant rate of loss of absorbance was seen. This slow rate is probably due to additional decomposition of BrKG occurring in the assay buffer which produces a substrate for the enzyme. After extrapolation to zero time, the loss in 340-nm absorbance (6.22 mM^{-1} cm^{-1}) yields the amount of glutamate dehydrogenase substrate in the aliquot of the sample.

Kinetics of Reaction of 3,4-Didehydro-2-ketoglutarate with DPN–Isocitrate Dehydrogenase. 3,4-Didehydro-2-ketoglutarate (eneKG) was prepared by a 10-h incubation of BrKG (10 mM) at pH 6.1 in buffer A. A stock solution of BrKG (110 mM, free acid) was added to buffer at pH 7.85 in order to obtain a final pH of 6.0. On the basis of the kinetics of bromide loss ($t_{1/2}$ = 1.6 h), only 1.4% of the original BrKG will remain after 10 h. The amount of GSH reactive compound is attributed (after a small correction for BrKG remaining) to eneKG (3.6 mM). Samples were used immediately without storage unless indicated.[2] The inactivation reactions were started by addition of DPN–isocitrate dehydrogenase (0.2 mg/mL) to this solution of eneKG. Any added ligands, as indicated, were also added to the eneKG solution. Two parallel incubations were always run: one with eneKG (experimental) and one without eneKG (control). Aliquots (5 μL) were withdrawn at given time intervals and assayed for activity by each of the following assays at 25 °C: (1) "standard assay", which was 20 mM isocitrate, 1 mM DPN, and 2 mM $MnSO_4$ in Tris–33 mM acetate buffer, pH 7.2; (2) "low isocitrate", which contained 0.5 mM isocitrate, 1 mM DPN, and 1 mM $MnSO_4$ in triethanolamine chloride buffer (36 mM in chloride) (pH 7.0); (3) "ADP", which consisted of the low isocitrate solution plus 2 mM ADP. The activity of the experimental incubation (E) was divided by the activity of the control incubation (E_c) to correct for any small activity loss not due to eneKG. The rate constant for inactivation (k_{inact}) was obtained by a linear least-squares fit to ln $[E/E_c]$ vs. time. 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. R is the ratio of activity with ADP (assay 3) to the activity without ADP (assay 2) in the experimental incubation mixture. R_0 is approximately 4.7 and R_∞ is taken as 1.0. The uncertainties in the rate constants are expressed at the 90% confidence limit and are calculated by the method of Blaedel & Iverson (1976).

Determination of the Stoichiometry of Reagent Incorporation. DPN–isocitrate dehydrogenase was incubated with 3.6 mM [^{14}C]eneKG at 25 °C in buffer A, pH 6.1, and loss of activity was followed as described above. At selected times, aliquots were removed from both the experimental and control incubations. Excess reagent was removed by the gel filtration method described previously (Bednar et al., 1982). The specific activities and stoichiometry of reagent incorporated were determined by the same method described previously (Bednar et al., 1982).

Determination of the Identity of the Modified Residue. DPN–isocitrate dehydrogenase was modified with [^{14}C]BrKG (Bednar et al., 1982) or with [^{14}C]eneKG. After the incorporation was determined, modified enzyme was dialyzed for 8 h against two changes of 6 L of water. The modified enzyme was transferred to an amino acid analysis tube and made 3% in hydrogen peroxide. After the sample was kept overnight in the refrigerator, 140 μL of 48% HBr was added. After 0.5 h, the sample was evaporated to dryness and hydrolyzed in vacuo using 6 N HCl at 110 °C for 20 h. The hydrolysate was dried and dissolved in water. An aliquot (1/20) was counted in a liquid scintillation counter while the rest was diluted with 0.2 N sodium citrate buffer, pH 2.2, and quantitatively applied to a Beckman Model 120C amino acid analyzer. The 55-cm column (AA15 ion-exchange resin) was eluted with 0.2 N sodium citrate buffer, pH 3.25, for 100 min. The buffer was changed to 0.2 N sodium citrate buffer, pH 4.23, for another 100 min. Fractions of the effluent were collected after passage through the photometer at 1.0-min (1.7 mL) intervals. Radioactivity was measured in a liquid scintillation counter at 4 °C after addition of 0.1 mL of concentrated HCl and 10 mL of ACS to 1.0 mL of each fraction. Elution position of radioactive peaks obtained from modified protein was compared with those of standards. Standards were prepared by reacting both [^{14}C]BrKG and [^{14}C]eneKG with equimolar amounts of GSH (15 mM) at pH 6–8 in water for 15 min. Amino acid analysis of this reaction mixture revealed the absence of unreacted GSH. The product was treated with hydrogen peroxide and hydrolyzed in the same manner as described above for modified protein.

[2] If aliquots of this solution were stored frozen at –80 °C, a somewhat greater (20–30%) rate of inactivation was observed.

S-(*N*-Ethylsuccinimido)cysteine was prepared by overnight reaction of *N*-ethylmaleimide (10 μmol) with *N*-acetylcysteine (10 μmol) at pH 5.6 (Smyth et al., 1960). The adduct was then hydrolyzed in 6 N HCl at 110 °C for 3 days to yield *S*-succinylcysteine (Smyth et al., 1961). After evaporation in vacuo, an aliquot (40 nmol) was loaded on an amino acid analyzer, and the elution position of the ninhydrin-positive succinylcysteine was determined. Another aliquot (40 nmol) was treated with hydrogen peroxide and hydrolyzed as described above. When this sample was loaded onto the analyzer, no ninhydrin-positive peaks could be seen.

Modified enzyme of known covalent incorporation was also treated with 0.1 M sodium borohydride at pH 10 for 30 min and then dialyzed against two changes of 6 L of water for 18 h. The protein was then hydrolyzed (6 N HCl, 110 °C, 18 h) and subjected to amino acid analysis. The elution profile of radioactive components was determined. Standards were prepared by reacting [^{14}C]BrKG (or [^{14}C]eneKG) (15 mM) with a slight excess of cysteine (or GSH) at pH 6–8 in water for 15 min. The products were then treated with sodium borohydride as described above. The GSH product was purified by ion-exchange chromatography. The glutathione derivative emerged from an AG-50 column (1.0 × 40 cm) after elution with 130 mL of 0.05 N HCl. After treatment of this product with 6 N HCl for 20 h at 110 °C, the sample was subjected to amino acid analysis. The cysteine product was analyzed directly. The 27-min peak is ninhydrin positive with a color constant 61% that of aspartic acid.

N-Acetylhistidine (1 M) was reacted with [^{14}C]BrKG (5 mM) at pH 10.4 for 24 h at room temperature. *N*-Acetyllysine (1 M) was reacted with [^{14}C]BrKG (0.5 mM) at pH 10 for 24 h. [^{14}C]BrKG (0.5 mM) was titrated to pH 9 and allowed to stand for 24 h. These three samples were then treated with 0.1 M sodium borohydride for 30 min, titrated to pH <1 with 6 N HCl, and evaporated to dryness in vacuo. After 24-h hydrolysis in 6 N HCl at 110 °C, each sample was separately loaded on an amino acid analyzer, and the elution profiles of radioactivity were determined. The *N*-acetylhistidine/[^{14}C]BrKG reaction yielded three radioactive peaks (50, 79, and 152 min), in addition to peaks due to the decomposition of BrKG alone. The *N*-acetyllysine/[^{14}C]BrKG reaction yielded peaks at 83 and 149 min. The peaks of the lysine and histidine derivatives were isolated from an amino acid analyzer (before reaction with ninhydrin) and subjected to a 24-h hydrolysis in 6 N HCl at 110 °C. Rerunning these samples on the amino acid analyzer yielded the same peaks without decomposition into components which eluted earlier than aspartic acid.

Results and Discussion

Effect of Reagent Preincubation. Aliquots of BrKG, which were preincubated in the incubation mixture (buffer A) for various periods of time, were added to DPN-dependent isocitrate dehydrogenase, and the rate of enzyme inactivation (k_{inact}) was measured. Figure 1 shows that a preincubation of several hours resulted in an increase in the inactivation rate, suggesting the production of a more effective inactivator. The subsequent decrease in the rate of inactivation with further preincubation implies that this inactivator is also unstable.

Minimal Decomposition Scheme for BrKG. A variety of chemical parameters were followed in order to characterize kinetically the decomposition of BrKG. Figure 2A shows that loss of reactivity toward glutathione can be fit with a half-time of 7 h. However, the loss of bromide is much faster than the loss of ability to react with a nucleophile. BrKG loses bromide with a half-time of 1.6 h (Figure 2B). The loss of a substrate

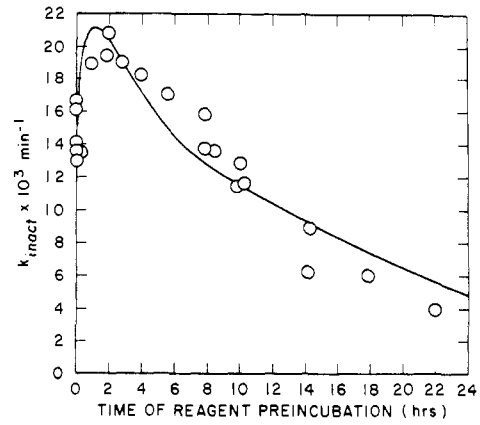

FIGURE 1: Rate constant for inactivation of DPN-dependent isocitrate dehydrogenase as a function of time of preincubation of 3-bromo-2-ketoglutarate (BrKG). BrKG was preincubated at 25 °C in 50 mM Mes buffer (pH 6.1) containing 2 mM $MnSO_4$ and 20% glycerol (buffer A). At the indicated times an aliquot was added to a solution of DPN–isocitrate dehydrogenase. The loss of enzyme activity was followed by the standard assay. The rate constant was obtained by a linear least-squares fit of ln E/E_c vs. time for the first 36 min. The line is a theoretical line based on a model described under Results and Discussion.

for TPN–isocitrate dehydrogenase (Figure 2C) can also be fit to a half-time of 1.6 h over the first 1.5-h period. Longer incubations show the appearance of another substrate for TPN–isocitrate dehydrogenase. The presence of a minimum at about 4 h suggests that this new substrate must be separated from BrKG by at least one kinetically significant intermediate. The appearance of a substrate for bovine liver glutamate dehydrogenase can also be followed with time (Figure 2D). The glutamate dehydrogenase substrate is likely to be identical with the new substrate which appears for TPN–isocitrate dehydrogenase. Assaying with glutamate dehydrogenase first leaves no TPN–isocitrate dehydrogenase substrate. Assaying with TPN–isocitrate dehydrogenase first still yields 50% of the amount of the glutamate dehydrogenase substrate. This result suggests that TPN–isocitrate dehydrogenase uses only one isomer, while glutamate dehydrogenase uses both isomers of the same compound. The elimination of HBr without loss of ability to add nucleophile suggests the formation of the unsaturated compound 3,4-didehydro-2-ketoglutarate (eneKG). This unsaturated compound would be electrophilic and could add nucleophiles at C-4 by a Michael-type addition.

A minimal decomposition scheme for BrKG is presented in Figure 3. The formation of bromide (Figure 2B) and the loss of a substrate for TPN–isocitrate dehydrogenase (Figure 2C) were used to obtain the rate of loss of BrKG ($t_{1/2}$ = 1.6 h). The loss of glutathione reactive compounds (BrKG and eneKG) appears to be pseudo first order over the entire incubation. This result indicates that BrKG and eneKG add solvent (vertical arrows) at the same rate ($t_{1/2}$ = 7 h). Glutamate dehydrogenase probably utilizes both isomers of 4-hydroxy-2-ketoglutarate (C) while TPN–isocitrate dehydrogenase utilizes only one isomer. The lines in Figure 2 represent theoretical lines based on the minimal decomposition scheme and the assigned rate constants. The scheme clearly fits the loss of reactivity toward GSH (Figure 2A) and the loss of bromide ion (Figure 2B). Some deviation occurs at long times for the appearance of the substrates for TPN–isocitrate dehydrogenase and glutamate dehydrogenase (Figure 2C,D). Some decarboxylation which occurs over long time periods may explain this deviation. By 22 h, 14 mol % CO_2 was released; this corresponds to 0.7 mM decarboxylated compound(s).

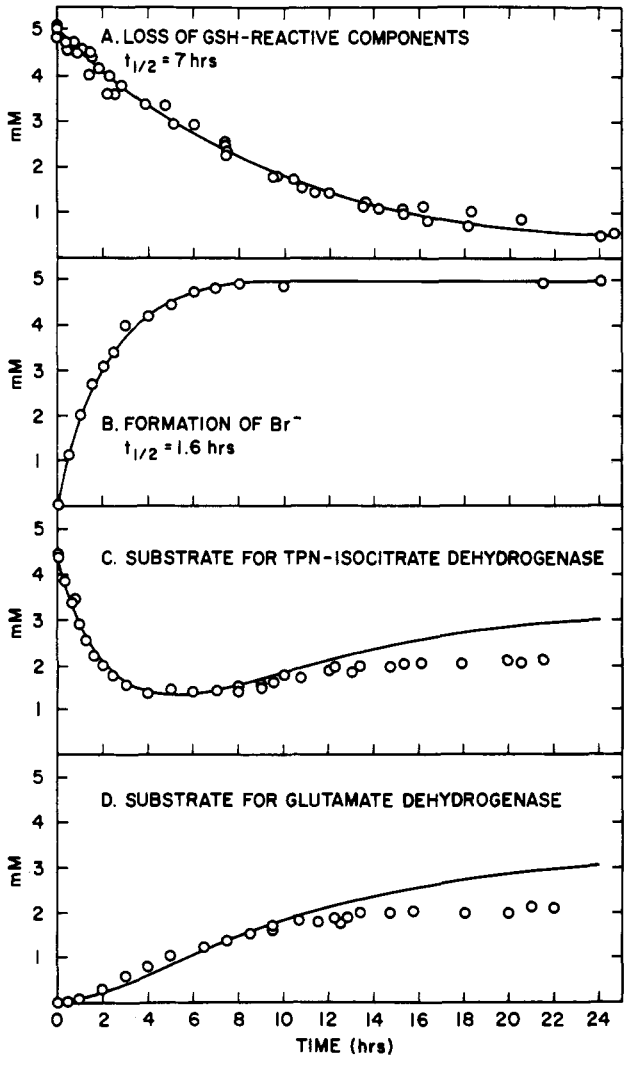

FIGURE 2: Decomposition of 3-bromo-2-ketoglutarate. BrKG (4.5–5.0 mM) was incubated at pH 6.1 in buffer A, and the concentrations of various species were determined over a 24-h period as described under Experimental Procedures. (A) Concentration of glutathione reactive compounds, i.e., BrKG (A) and eneKG (B). The solid line is given by the equation $[A + B]_t = [A_0/(k_C - k_B - k_D)][(k_C - k_D)e^{-(k_B+k_D)t} - k_B e^{-k_C t}]$. (B) Concentration of bromide ion produced. Solid line is given by the equation $[Br^-]_t = A_0(1 - e^{-(k_B+k_D)t})$. (C) Twice the concentration of substrate for TPN–isocitrate dehydrogenase. Both BrKG (A) and 4-hydroxy-2-ketoglutarate (C) appear to be substrates. The solid line is $A_t + C_t$ where $A_t = A_0 e^{-(k_B+k_D)t}$ and $C_t = [A_0 k_B/[(k_C - k_B - k_D)(k_B + k_D)]][k_B + k_D e^{-k_C t} - e^{-(k_B+k_D)t} - k_B - k_D + k_C]$. (D) Concentration of substrate for glutamate dehydrogenase. 4-Hydroxy-2-ketoglutarate (C) is assumed to be the substrate. The solid line is given by the equation for C_t given in (C). The rate constants for the above equations are given in Figure 3.

Buffer has a significant effect on the stability of BrKG. The decomposition rate of BrKG is less at pH 8.0 without buffer ($t_{1/2}$ = 2 h) than it is at pH 6.0 in the presence of the 50 mM Mes buffer used for the inactivation experiments. There is also a significant buffer effect on the loss of GSH reactivity. In the 50 mM Mes buffer, a maximum half-time of 8.2 h is predicted at low pH.[3] However, in the absence of buffer (pH 2.5) a half-time of 7.4 days is observed.

Is the Decomposition of BrKG or eneKG Enzyme Catalyzed? The decomposition rates were determined in the presence of enzyme, under the same conditions described under Experimental Procedures for the inactivation reaction,[4] to test if the DPN-dependent isocitrate dehydrogenase might catalyze the decomposition of BrKG or eneKG. The loss of bromide occurs with a half-time of 1.5 h in the presence of enzyme. This is not significantly different from the 1.6-h half-time in the absence of enzyme. The loss of GSH reactivity occurs with a half-time of 6.9 h in the presence of enzyme. This is within the experimental error of 7 h observed in the absence of enzyme. These results indicate that the DPN-dependent isocitrate dehydrogenase does not measurably catalyze the decomposition of either BrKG or eneKG.[5]

Kinetics of Reaction of eneKG with DPN–Isocitrate Dehydrogenase. Incubation of DPN-dependent isocitrate dehydrogenase with eneKG results in a rapid time-dependent loss of enzyme activity. Semilog plots curve after about 60% activity is lost (Figure 4). However, essentially all of the activity can be lost. The lack of linearity of the semilog plots below 40% residual activity is not due to reagent depletion; the degree of curvature is much more than can be explained by a 7-h half-time for the loss of eneKG. The curvature may be due to negative cooperativity in the reaction of eneKG with the multiple enzyme subunits. Activity is not restored by gel filtration. A rate saturation effect is observed upon plotting the reciprocal of the initial inactivation rate constant as a function of the reciprocal of the reagent concentration (Figure 5). The calculated K_{inact} (the reagent concentration yielding half the maximal rate of inactivation for eneKG) is 1.5 mM. The k_{max} (first-order rate constant at infinite reagent concentration) is 0.020 min^{-1}, which corresponds to a minimal half-time (T_{min}) of 35 min. These data are consistent with one of the criteria of an affinity label, i.e., prior binding of reagent before covalent bond formation.

The effect of reagent preincubation shown in Figure 1 can be explained quantitatively. The rate of inactivation at any time is the sum of the inactivation rate from BrKG and eneKG. Since a rate saturation is observed with both reagents the equation for the instantaneous k_{inact} at any time t is

$$k_{inact}(t) = \frac{[BrKG]_t k_{max,BrKG}}{K_{inact,BrKG} + [BrKG]_t} + \frac{[eneKG]_t k_{max,eneKG}}{K_{inact,eneKG} + [eneKG]_t} \quad (1)$$

The line in Figure 1 is a theoretical line. The K_{inact} of 6.2 mM and a k_{max} of 0.034 min^{-1} were used for BrKG (Bednar et al., 1982). The K_{inact} and k_{max} for eneKG (see above) were used and the concentrations of BrKG(t) and eneKG(t) were calculated from the rate constants for the minimal decomposition scheme (Figure 3). The line represents a reasonable fit to the data. The slower rate constants (points below the line) at long times may be due to some protection by the decomposition products of BrKG and eneKG.

The reason eneKG appears to be a more effective inactivator than BrKG is that eneKG is bound more tightly by the enzyme (lower K_{inact} than BrKG) rather than that it has a faster maximum rate of inactivation (k_{max}).

In contrast to BrKG, the enzyme does not catalyze the reduction of eneKG.[6] It may be that an electron dense group

[3] Over the pH range 5.4–7 in Mes and Pipes buffer, the loss of glutathione reactivity (k_{GSH}) can be fit to the equation $k_{GSH}(min^{-1})$ = 15000[OH^-] + 0.0014.

[4] Since the concentration of enzyme subunits is only 5 μM and BrKG is present at 5 mM concentration, the loss of reagent due to covalent bond formation will not affect significantly the overall rate of the reagent decomposition.

[5] If a 4% increase in decomposition rate occurred but was undetected, the rate of conversion on the enzyme surface would be less than 0.5 min^{-1}. Therefore, the enzyme cannot increase the rate of decomposition by more than 2 orders of magnitude.

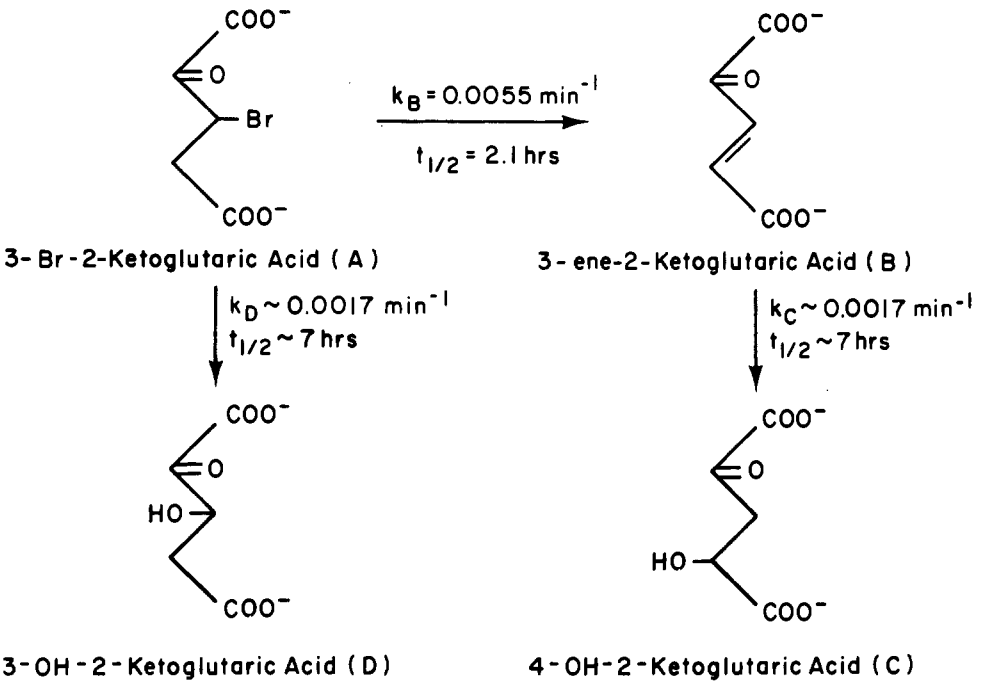

FIGURE 3: Minimal decomposition scheme for 3-bromo-2-ketoglutarate. 3-Bromo-2-ketoglutarate (A) can eliminate HBr to yield the conjugated compound 3,4-didehydro-2-ketoglutarate (B). 3,4-Didehydro-2-ketoglutarate would be expected to add nucleophiles (or solvent) by a Michael-type addition to C-4. The vertical arrows show decomposition of A and B by the action of solvent.

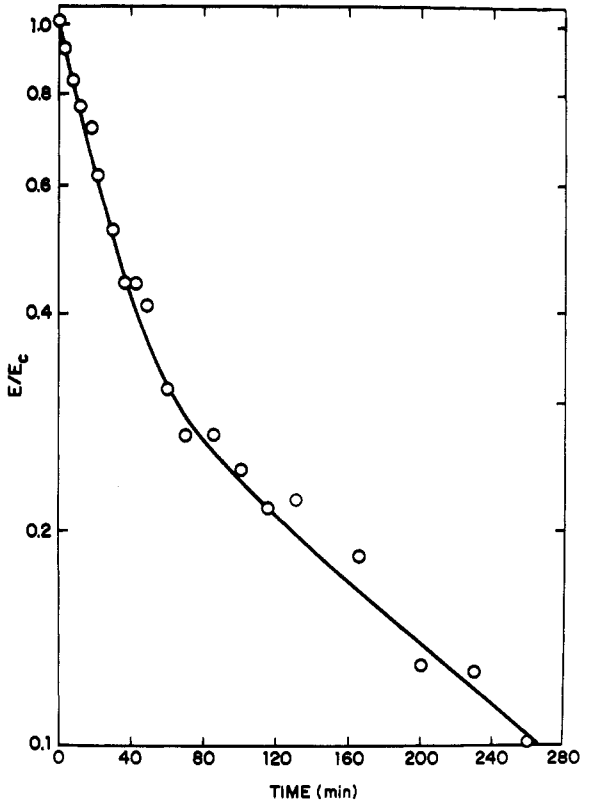

FIGURE 4: Time-dependent loss of DPN–isocitrate dehydrogenase activity on incubation with 3,4-didehydro-2-ketoglutarate. DPN–isocitrate dehydrogenase (0.2 mg/mL) was incubated at 25 °C with 3,4-didehydro-2-ketoglutarate (3.6 mM) at pH 6.1 in buffer A under the conditions described under Experimental Procedures. A plot of ln E/E_c vs. time is shown. E and E_c are the enzymatic activities measured by the standard assay in the experimental and control incubations, respectively. The rate constants for inactivation were obtained by a linear least-squares fit to the initial linear region of the curve (0–36 min).

(COO⁻ or Br) is required at C-3 for the enzyme to appreciably catalyze the reduction of the 2-keto group. Alternatively it

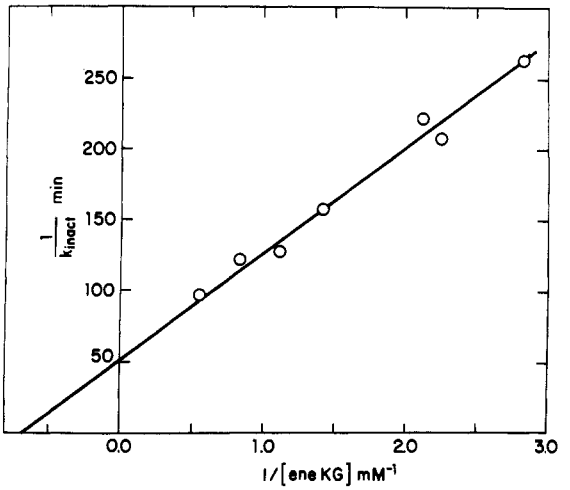

FIGURE 5: Dependence of the rate of inactivation on eneKG concentration. The rates of inactivation at different eneKG concentrations were calculated from a linear least-squares fit of ln E/E_c vs. time for t = 0–36 min. K_{inact} and k_{max} were calculated in accordance with the equation $1/k_{inact} = (K_{inact}/k_{max})(1/[\text{eneKG}]) + 1/k_{max}$.

is possible that the different geometry of eneKG militates against productive binding at the active site.

Effect of Added Ligands on the Inactivation of DPN–Isocitrate Dehydrogenase by 3,4-Didehydro-2-ketoglutarate. Isocitrate affords a high degree of protection of DPN–isocitrate dehydrogenase against inactivation by eneKG (Table I, line 2). A plot of the reciprocal of the inactivation rate constant as a function of isocitrate concentration is shown in Figure 6. With the assumption that isocitrate and eneKG are linear competitive inhibitors, a dissociation constant of 180 μM was calculated for total DL-isocitrate from the site at which it

[6] If eneKG were a substrate for DPN–isocitrate dehydrogenase, an upper limit on the rate of this reaction would correspond to a V_{max} of 0.09 unit/mg (k_{cat} = 4 min⁻¹) assuming the K_m is equal to the K_{inact} of 1.5 mM. For comparison, a V_{max} of 8 units/mg (k_{cat} = 320 min⁻¹) and a K_m of 5.2 mM were measured for BrKG (Bednar et al., 1982).

Table I: Effect of Ligands on the Rate of Inactivation by eneKG[a]

additions to reaction mixture	$k_{inact}(+L)/k_{inact}(-L)$	K_i (μM)
(1) none	1.00	
(2) isocitrate (20 mM)	0.09	
(3) 2-ketoglutarate (110 mM)	0.92	
(4) DPN (0.5 mM)	0.47	220
(5) DPN (5.0 mM)	0.15	440
(6) DPNH (0.5 mM)	0.41	175
(7) DPNH (5.0 mM)	0.22	700
(8) ADP (0.1 mM)	0.70	1.5[b]
(9) ADP (0.3 mM)	0.27	1.3[b]
(10) ADP (1.0 mM)	0.17	2.1[b]

[a] DPN-isocitrate dehydrogenase was incubated with 3,4-didehydro-2-ketoglutarate (eneKG), $MnSO_4$ (2 mM), and the indicated ligands (L) at 25 °C in buffer A, pH 6.1. [eneKG]/K_{inact} = 1.0. K_i is defined by the equation $k_{inact}(+L)/k_{inact}(-L) = (1 + [eneKG]/K_{inact})/(1 + [eneKG]/K_{inact} + L/K_i)$. [b] For ADP^{3-}.

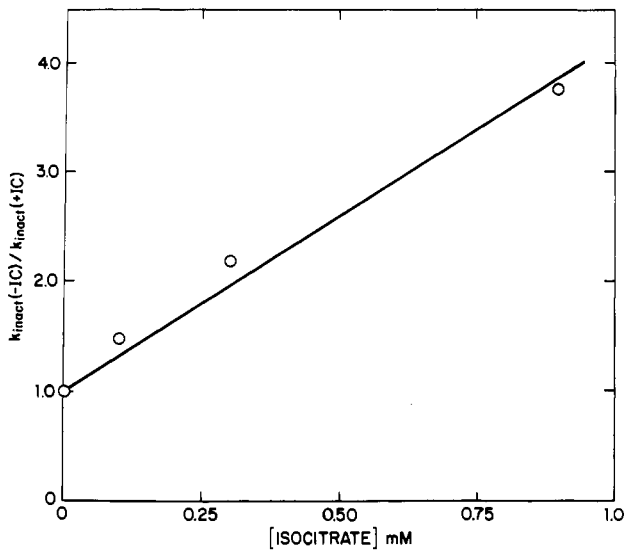

FIGURE 6: Isocitrate protection of DPN-isocitrate dehydrogenase from 3,4-didehydro-2-ketoglutarate inactivation. The rate constants for inactivation at the indicated concentrations of isocitrate (IC) and 2 mM $MnSO_4$ were calculated from a linear least-squares fit of ln E/E_c vs. time. K_{IC} (the inhibition constant for isocitrate from the site at which it protects) was calculated from the equation $k(-IC)/k(+IC) = K_{inact}[IC]/[K_{IC}(K_{inact} + [eneKG])] + 1$ using $[eneKG] = K_{inact}$.

protects. This constant can be compared with a directly determined binding constant of 320 μM for total DL-isocitrate at the active site (Ehrlich & Colman, 1981). Isocitrate thus appears to protect by a specific interaction with the active site.

2-Ketoglutarate (Table I, line 3) does not offer significant protection. This observation is likely to be due to very weak binding of 2-ketoglutarate to free enzyme, as indicated by Bednar et al. (1982). The protection offered by coenzymes DPN and DPNH (Table I, lines 4–7), while significant, is much less that that expected based on the directly determined binding constants of 55 μM (Ehrlich & Colman, 1981) and 2.8 μM (Ehrlich & Colman, 1982) for DPN and DPNH, respectively. Furthermore, a constant value of K_i is not obtained as the concentrations of DPN or DPNH are varied (Table I). The observed protection is therefore unlikely to be caused by a specific interaction with the coenzyme sites.

The activator ADP does offer significant protection which is reasonably consistent with its directly determined binding constant (Ehrlich & Colman, 1981). With the assumption that ADP and eneKG are competitive, an average binding constant for ADP (K_{ADP}) of 1.9 μM ADP^{3-} can be calculated

Table II: Rates of Loss of Activity (k_{inact}) and of ADP Activation (k_{ADP})[a]

additions	$k_{inact} \times 10^3$ (min^{-1})	$k_{ADP} \times 10^3$ (min^{-1})
(1) none	17.5 ± 2.5	18.2 ± 4.0
(2) isocitrate (0.9 mM)	4.0 ± 1.0	3.9 ± 2.7
(3) ADP (0.3 mM)	4.1 ± 1.0	4.8 ± 1.7

[a] DPN-isocitrate dehydrogenase was incubated with eneKG (3.6 mM), $MnSO_4$ (2 mM), and the indicated ligands at 25 °C in buffer A, pH 6.1. The loss of activity and loss of ADP activation were measured as described under Experimental Procedures.

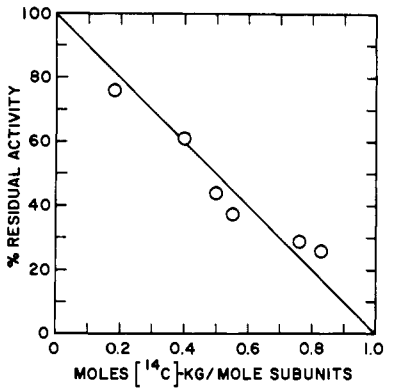

FIGURE 7: Residual activity as a function of incorporation for the reaction of DPN-isocitrate dehydrogenase with 3,4-didehydro-2-ketoglutarate. At selected times, aliquots of the reaction mixture were removed, and the specific activity and amount of reagent covalently incorporated were measured as described under Experimental Procedures.

from the data in Table I (lines 8–10). This value can be compared to a 2.6 μM ADP^{3-} binding constant obtained by Ehrlich & Colman (1981). It appears that protection by ADP is mediated by a specific interaction with the enzyme. However, it is likely that the protection is not due to a direct steric block but rather to an interaction between the ADP site and the active site. There is good evidence for interaction between the ADP and the isocitrate binding sites on this enzyme [see Discussion of Bednar et al. (1982)].

Loss of ADP Activation. Incubation of eneKG with DPN-isocitrate dehydrogenase also results in a time-dependent loss of ADP activation. The allosteric activation by ADP is observed as an increase in the initial velocity caused when the nucleotide is added to assay solution containing nonsaturating concentrations of isocitrate (Hayman & Colman, 1978). The rate constant for loss of ADP activation (k_{ADP}) was determined as described under Experimental Procedures. Table II (line 1) shows that the rate constant for loss of ADP activation is essentially the same as the rate constant for loss of activity (k_{inact}). This equality is also seen when both rates are slowed by the addition of isocitrate or ADP (Table II, lines 2 and 3). The equality of rate constants for loss of activity and loss of ADP activation suggests that both effects are the result of a single 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. Therefore the effect of eneKG on the ADP activation is likely to result from interaction between modified and unmodified subunits.

Characteristics of Covalent Incorporation into DPN-Isocitrate Dehydrogenase. The loss of activity is related to the moles of covalently bound reagent per subunit in Figure 7. Extrapolation to 0% residual activity indicates that 1.0 mol of [^{14}C]KG per enzyme subunit is incorporated when the enzyme is completely inactive. This plot suggests that the

modification of no more than one amino acid residue per average subunit is responsible for the loss of enzyme activity. Further, it suggests that each subunit potentially has an active site and that complete inactivation is not achieved until, on average, all subunits are modified; modification of one subunit does not cause inactivation of unmodified subunits.

Direct binding experiments by Ehrlich & Colman (1981, 1982) have revealed 0.5 binding site/enzyme subunit for Mn^{2+}, DPN, DPNH, TPNH, and metal-dependent binding of isocitrate and the activator ADP. eneKG may not behave as the normal substrates since 1.0 mol/subunit is bound for complete inactivation. However, in the absence of metal, isocitrate and ADP bind weakly to the enzyme with stoichiometries of 1/subunit or greater (Ehrlich & Colman, 1981). It is possible that the 0.5/subunit stoichiometry for the natural ligands in the binding studies could result from an extreme case of negative cooperativity between subunits or from a "flip-flop" type mechanism (Harada & Wolfe, 1968). In this case, it is not surprising that a compound which can form an irreversible bond to the enzyme would, given sufficient time, bind to all subunits. Consistent with this postulate is the observed decrease in the inactivation rate after 60% inactivation, suggesting that reaction with subunits in partially modified enzyme may proceed at a slower rate.

Identification of Modified Residue for BrKG and eneKG Inactivation. One of the difficulties in using halo ketone affinity labels is the necessity of identifying the derivatized amino acids for each new reagent. This contrasts with haloacetyl derivatives (Wilchek & Givol, 1977) which can be converted to easily identified carboxymethyl amino acids. A further complication in this work is that the products of reaction of amino acids with BrKG and eneKG would be structural isomers. The site of the amino acid linkage would be C-3 and C-4, respectively. For elimination of the need for separate standards for each reagent, an attempt was made to convert these C-3 and C-4 structural isomers into a single compound. Peroxide treatment of the 2-ketoglutaric acid derivatives would be expected to cause decarboxylation to a single succinic acid derivative. The amino acid linkage site would be C-2 and C-3 in the succinic acid derivative, but these are not distinguishable because of the symmetry of succinic acid. A procedure similar to this was used by Meloche (1970) to convert the GSH derivative of bromopyruvate to an easily detected carboxymethyl derivative. This type of procedure has the potential of simplifying the problem of identifying amino acids modified by BrKG and eneKG.

Of the 20 amino acids, at least 8 contain potentially nucleophilic side chains. The magnitude of the problem of identifying a modified residue can be reduced since the side chains of cysteine, lysine, and histidine are the most reactive and the most likely to react with a chemical modification reagent. Initially these would be the prime candidates for reaction with BrKG and eneKG. Succinic acid derivatives of an amino acid are the ultimate hydrolysis products of the adduct formed between amino acids and the common chemical modification reagent *N*-ethylmaleimide. The elution positions on an amino acid analyzer of the succinic acid derivatives of cysteine, lysine, and histidine are known (Brewer & Riehm, 1967). These positions provide a framework which can be used to identify residues modified by either BrKG or eneKG.

Modified protein was treated with hydrogen peroxide and loss of one-fifth of the total ^{14}C label was consistent with the decarboxylation of the 2-ketoglutarate derivatives. The elution position of ^{14}C label on an amino acid analyzer from BrKG and eneKG modified protein is shown in parts A and B of

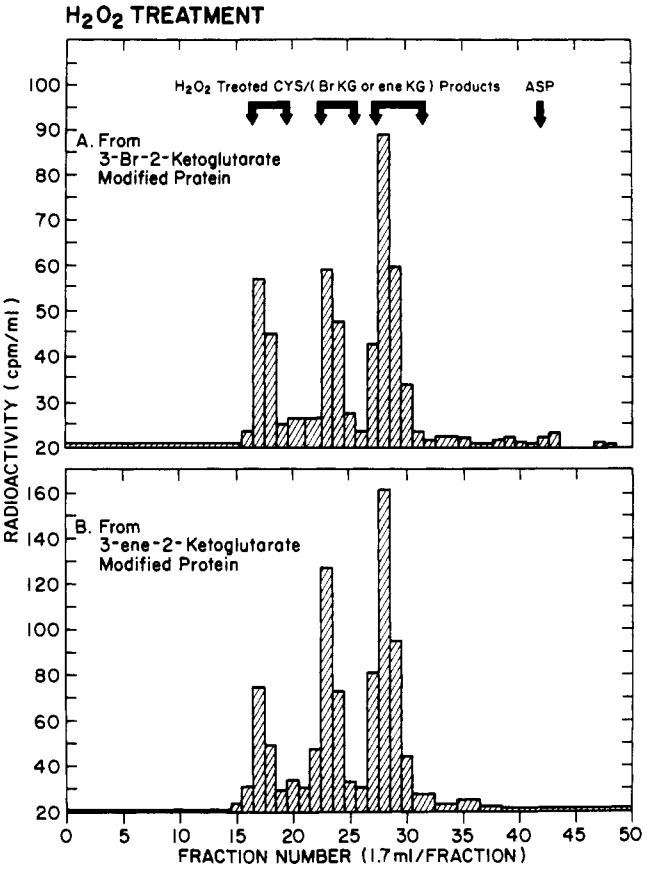

FIGURE 8: Amino acid analysis of peroxide-treated modified enzyme. ^{14}C-Modified enzyme was treated with hydrogen peroxide and hydrolyzed in 6 N HCl. (A) The elution pattern of ^{14}C label from BrKG modified protein. (B) The elution pattern of ^{14}C label from eneKG modified protein. The elution position of the GSH adduct of BrKG or eneKG after peroxide treatment and hydrolysis is shown by the bold arrows labeled "H_2O_2 Treated Cys/(BrKG or eneKG) Products". The elution position of aspartic acid (42 min) is shown for reference.

Figure 8, respectively. The striking similarity of the elution positions suggests that the same type of residue(s) is modified by both reagents. Histidine is not modified since the two succinic acid derivatives of histidine elute just before and after aspartic acid (42 min) (Brewer & Riehm, 1967). The lysine derivatives would be expected to elute at approximately 78 min, which is after glutamic acid (66 min) and before glycine (90 min) (Brewer & Riehm, 1967). However, fractions up to 200 min were counted, and no radioactivity was found beyond 35 min. The 29-min peak corresponds to the known elution position of succinylcysteine, indicating that cysteine may be modified. The multiple peaks may be due to the modification of three types of residues; the stoichiometry of the inactivation makes this unlikely, but by no means impossible. It is probable that the extra peaks result from the decomposition of succinylcysteine. The peroxide treatment may have caused oxidation of the sulfur to the sulfoxide and the sulfone. Indeed, we found succinylcysteine to be unstable to the conditions used for the peroxide treatment and subsequent hydrolysis.

A standard was prepared by reacting BrKG with GSH to confirm the elution position of the decomposition of a modified cysteine. The adduct was treated with peroxide and then subjected to acid hydrolysis by the same procedure used with the modified protein. The standards also exhibited three peaks which eluted at the same times as the radioactivity from the modified proteins (see arrows in Figure 8). These results make cysteine the prime candidate for the amino acid residue modified by both reagents.

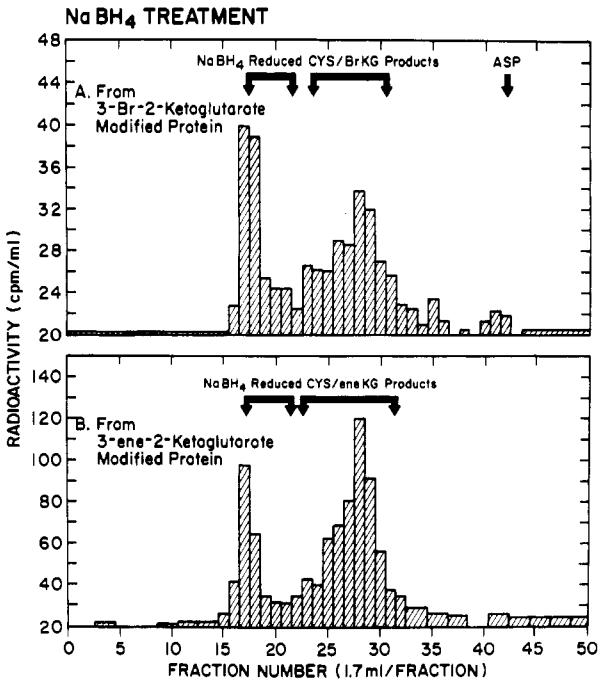

FIGURE 9: Amino acid analysis of sodium borohydride reduced modified enzyme. ^{14}C-Modified enzyme was treated with sodium borohydride and acid hydrolyzed. The elution position of ^{14}C labeled from BrKG- and eneKG-modified protein is shown in (A) and (B), respectively. The elution position of standards prepared by reacting cysteine with the appropriate reagent followed by sodium borohydride treatment is shown by the bold arrows. The elution position of aspartic acid (42 min) is shown for reference.

The α-halo ketones react with protein nucleophiles to produce radioactive products which may be susceptible to further nucleophilic attack, since they remain carbonyl compounds possessing a leaving group α to the carbonyl group. It is possible that label migration may have occurred during the peroxide treatment. A thiol group, being a good nucleophile, may have displaced the originally modified residue. In modification of triosephosphate isomerase with haloacetyl phosphate (de la Mare et al., 1972), the migration of the phosphorylhydroxyacetonyl group from a Glu to a Tyr occurs.

The covalently incorporated ketone was chemically reduced with $NaBH_4$ to guard against the possibility of label migration. The protein was subsequently hydrolyzed, and the amino acids were separated on an amino acid analyzer. Standards were prepared by reacting separately BrKG and eneKG with the -SH of the Cys moiety in GSH or the amino acid Cys itself. The resulting products were treated in a manner similar to that from the protein. Figure 9 compares the elution position of the ^{14}C label for both BrKG- and eneKG-modified protein with their appropriate standards. The reason for the multiple peaks in the protein and standards is not understood. However, the reasonable agreement of the elution times with the standards is consistent with the conclusion that a cysteine is the amino acid modified by both reagents. The borohydride-reduced derivatives of lysine and histidine with the reagents elute much later than aspartic acid, and these derivatives do not decompose into peaks which elute before aspartic acid (as described under Experimental Procedures). It appears that the original site of modification must be cysteine, unless label migration occurs prior to the chemical reduction of the ketone.

Conclusion

The decomposition product of 3-bromo-2-ketoglutarate, presumed to be 3,4-didehydro-2-ketoglutarate, like its parent compound, appears to fulfill many of the criteria of an affinity label for the active site of DPN-isocitrate dehydrogenase: (1) structural analogy to normal substrate, (2) reversible binding prior to irreversible inactivation, (3) specific protection by isocitrate, and (4) stoichiometric inactivation. These affinity labels cause loss of the allosteric ADP activation at the same rate as loss of catalytic activity. The loss of ADP activation is likely to result from an interaction between inactive modified subunits and the active unmodified subunits. The only major difference between these two reagents is that eneKG is not catalytically reduced by the enzyme as is BrKG. An essential cysteine residue, which might function as a general base in the catalytic reaction mechanism, is modified by both these affinity labels.

3-Bromo-2-ketoglutarate and 3,4-didehydro-2-ketoglutarate both appear to be affinity labels of the active site of DPN-isocitrate dehydrogenase. Their structural analogy to 2-ketoglutarate and their high reactivity suggest that they may prove useful in probing the active sites of some of the many enzymes that utilize 2-ketoglutarate.

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