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Cysteinyl Peptide Labeled by 3-Bromo-2-ketoglutarate in the Active Site of Pig Heart NAD^+-Dependent Isocitrate Dehydrogenase†

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ABSTRACT: The substrate affinity label 3-bromo-2-ketoglutarate (BrKG) reacts covalently with pig heart NAD^+-specific isocitrate dehydrogenase with complete inactivation and incorporation of about 0.8 mol of reagent/mol of average enzyme subunit [Bednar, R. A., Hartman, F. C., & Colman, R. F. (1982) *Biochemistry 21*, 3681–3689]. Protection against inactivation is provided by isocitrate and Mn^{2+}. We have now identified a critical modified peptide by comparison of the peptides labeled by BrKG at pH 6.1 in the absence and presence of isocitrate and Mn^{2+}. Modified enzyme, isolated from unreacted BrKG, was incubated with [^{3}H]$NaBH_4$ to reduce the keto group of protein-bound 2-ketoglutarate and thereby introduce a radioactive tracer into the modified amino acid. Following carboxymethylation and digestion with trypsin, the specific modified peptide was isolated by reverse-phase HPLC, first in 0.1% trifluoroacetic acid with a gradient in acetonitrile and then in 20 mM ammonium acetate, pH 5.8, with an acetonitrile gradient. Gas-phase sequencing gave the modified peptide: Ser-Ala-X-Val-Pro-Val-Asp-Phe-Glu-Glu-Val-Val-Val-Ser-Ser-Asn-Ala-Asp-Glu-Glu-Asp-Ile-Arg. The corresponding tryptic peptide that was isolated from unmodified enzyme yielded the same sequence except for (carboxymethyl)cysteine at position 3, suggesting that cysteine is the target of 3-bromo-2-ketoglutarate. Pig heart NAD^+-dependent isocitrate dehydrogenase is composed of three distinct subunits (α, β, and γ) that can be separated by chromatofocusing in urea and identified by analytical gel isoelectric focusing. The peptide modified by 3-bromo-2-ketoglutarate, which is in or near the substrate site, is derived only from the separated γ subunit.

The pig heart NAD^+-specific isocitrate dehydrogenase [*threo*-D_S-isocitrate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.41] has been shown to be inactivated by covalent reaction with 3-bromo-2-ketoglutarate (BrKG),[1] which acts as an affinity label of the isocitrate binding site (Bednar et al., 1982a). A marked decrease in the rate constant for in-

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[1] Abbreviations: BrKG, 3-bromo-2-ketoglutarate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DTT, dithiothreitol; Cm, carboxymethyl; PTH, phenylthiohydantoin.

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activation was produced by isocitrate in the presence of Mn^{2+}, whereas the coenzymes NAD^+ and NADH were much less effective in preventing inactivation. Measurement of the amount of [^{14}C]BrKG incorporated into the enzyme as a function of inactivation yielded an extrapolated value at 0% residual activity of 0.83 mol of reagent/mol of average subunit, suggesting that modification of no more than one amino acid residue per average subunit is responsible for loss of enzyme activity. Evidence based on amino acid analysis of the acid-hydrolyzed modified enzyme indicated that cysteine was the prime candidate for the residue attacked by BrKG (Bednar et al., 1982b); however, no particular cysteine was identified. In this paper we report the isolation and characterization of a cysteine-containing peptide that is modified by 3-bromo-2-ketoglutarate in this NAD^+-dependent isocitrate dehydrogenase and is protected by isocitrate and Mn^{2+}.

The pig heart NAD^+-specific isocitrate dehydrogenase is a multisubunit enzyme with three nonidentical subunits present in the ratio $2\alpha:1\beta:1\gamma$ (Ramachandran & Colman, 1980; Colman, 1983). These subunits are characterized by similar molecular weights of about 40 000, but are distinguished by their isoelectric points. The smallest complete enzyme is a tetramer of molecular weight 160 000 (Ehrlich et al., 1981). The functions of the distinct subunits are not known. However, there is one divalent metal-dependent isocitrate site for every two subunits and one Mn^{2+} binding site per two subunits (Ehrlich & Colman, 1981). These observations raise the possibility that the subunits are specialized in function. It was previously reported that when [^{14}C]BrKG was used to modify the enzyme, radioactivity was found in every type of subunit, although the highest amount of label relative to the amount of protein was located in the γ subunit, and proportionately less radioactivity was associated with the α subunit (Bednar & Colman, 1982). In the present paper, we evaluate the subunit source of a critical peptide modified by 3-bromo-2-ketoglutarate and present evidence that it is derived from the γ subunit.

Experimental Procedures

Materials. NAD^+-dependent isocitrate dehydrogenase was purified from pig hearts as described by Ehrlich et al. (1981). The enzyme was dialyzed at 4 °C for 24 h against two changes of 50 mM Mes buffer, pH 6.6, containing 20% glycerol and 2 mM $MnSO_4$. It was stored in aliquots at −75 °C. The enzyme concentration was calculated from the absorbance at 280 nm by using $E^{1\%}_{280} = 6.45$ (Shen et al., 1974) and a value of 40 000 as the average subunit molecular weight (Ramachandran & Colman, 1980). The (*RS*)-3-bromo-2-ketoglutaric acid was synthesized by direct bromination of 2-ketoglutarate as described by Hartman (1981). The sample used in this study was a gift from Fred C. Hartman. A stock solution (110 mM) of 3-bromo-2-ketoglutarate (free acid) was prepared by dissolving a measured weight in distilled water as described by Bednar et al. (1982) and was stored in aliquots at −75 °C.

The mercaptoethanol, dithiothreitol, DL-isocitrate, NAD^+, sodium borohydride, bovine pancreas *N*-tosyl-L-phenylalanine chloromethyl ketone treated trypsin, and Sephadex G-50–80 were obtained from Sigma Chemical Co. Sodium [^{3}H]borohydride dissolved in 0.1 N NaOH was purchased from Amersham Corp. and [1-^{14}C]iodoacetic acid from New England Nuclear Corp. Pharmacia supplied the PBE-94 resin and buffers used for chromatofocusing, and the ampholytes and other chemicals required for the isoelectric focusing gels were obtained from Bio-Rad.

Preparation of Enzyme Modified by 3-Bromo-2-ketoglutarate. Isocitrate dehydrogenase (1 mg/mL) was preincubated for 20 min at 25 °C in 50 mM Mes buffer, pH 6.3, containing 20% glycerol and 2 mM $MnSO_4$. This pH was chosen so that after the addition of either BrKG or HCl the final reaction pH would be 6.1. The reaction period was initiated by the addition to the experimental sample of 3-bromo-2-ketoglutarate (to yield a final concentration of 5 mM) and to the control sample of HCl (to yield 10 mM). For some experiments (indicated under Results) 10 mM DL-isocitrate was included during the preincubation and reaction periods to protect against inactivation. Aliquots were withdrawn during the incubation period to assay the isocitrate dehydrogenase activity spectrophotometrically at 340 nm by using a 1.0-mL standard assay at 25 °C containing 1 mM NAD^+, 2 mM $MnSO_4$, and 20 mM DL-isocitrate in Tris–33 mM acetate, pH 7.2. After 150 min, the reaction mixture was cooled on ice, and the excess BrKG was removed by using the gel centrifugation procedure of Penefsky (1979). Each 0.5 mL of reaction mixture was applied to a 5-mL column of Sephadex G-50–80 equilibrated at 4 °C with 50 mM Mes buffer, pH 6.1, containing 20% glycerol, 2 mM $MnSO_4$, and 0.1 mM dithiothreitol.

Reduction of BrKG-Modified Enzyme by [^{3}H]$NaBH_4$. To introduce a radioactive label into nonradioactive BrKG-modified enzyme, the modified enzyme was treated with [^{3}H]$NaBH_4$ to reduce the keto group of the reagent that is covalently linked to the protein. The modified enzyme was reduced by two additions of 180 mM [^{3}H]$NaBH_4$ in 0.1 M NaOH (specific radioactivity 1.27×10^{12} cpm/mol of hydrogen), 20 min apart, to give a final concentration of 3 mM $NaBH_4$. Twenty minutes after the second addition, solid guanidine hydrochloride was added to yield a final concentration of 5 M, and the free sodium borohydride was removed by another gel centrifugation step, this time by use of Sephadex G-50–80 equilibrated with 50 mM Hepes buffer, pH 8.0, containing 5 M guanidine hydrochloride.

Carboxymethylation and Proteolytic Digestion of BrKG-Modified and Unmodified Enzymes. Dithiothreitol was added to the isocitrate dehydrogenase solution in 5 M guanidine hydrochloride in an amount 10 times the number of moles of cysteine in the enzyme (Ramachandran & Colman, 1980). The enzyme was incubated with dithiothreitol at room temperature for 1 h after which time iodoacetic acid was added in an amount 2.2 times the number of moles of dithiothreitol used. For certain experiments, as indicated under Results, nonradioactive sodium borohydride and [1-^{14}C]iodoacetate (specific radioactivity 2.4×10^{12} cpm/mol) were used in the same concentrations as described above. After the enzyme was incubated with iodoacetate at room temperature for 10 min, β-mercaptoethanol was added at 10 times the amount of iodoacetate, and the incubation was continued for another 10 min at room temperature. The enzyme samples were then dialyzed for 24 h at 4 °C against 50 mM ammonium bicarbonate, pH 8.0, with two changes. The enzyme was digested with *N*-tosyl-L-phenylalanine chloromethyl ketone treated trypsin, 1:100 (w/w) with respect to isocitrate dehydrogenase at 37 °C for 15 min, followed by the addition of a second equal amount of trypsin and incubation for another 15 min. Samples were then lyophilized to remove the ammonium bicarbonate prior to HPLC.

Separation of Tryptic Peptides by High-Performance Liquid Chromatography. The tryptic peptides from carboxymethylated enzymes were separated on a Vydac C-18 column (0.46 × 25 cm) by using a Varian Model 5000 HPLC system equipped with a Varichrom absorbance monitor. Two different

solvent systems were used. *Solvent System I*: The column was equilibrated with 0.1% trifluoroacetic acid in water (solvent A). After elution with solvent A for 10 min, a linear gradient was run to 35% solvent B (acetonitrile containing 0.07% trifluoroacetic acid) in 140 min, followed by a linear gradient to 60% solvent B in the next 20 min. In one set of experiments, as indicated under Results, a somewhat different gradient was used. *Solvent System II*: The column was equilibrated with 20 mM ammonium acetate, pH 5.8, in water (solvent C). After elution with solvent C for 10 min, a linear gradient was run to 60% solvent D (20 mM ammonium acetate in 50% acetonitrile) in 120 min, followed by a linear gradient to 100% solvent D in the next 30 min. In both systems the flow rate was 1 mL/min, the effluent was continuously monitored for absorbance at 220 nm, and fractions of 1 mL were collected. Aliquots of fractions were mixed with 10 mL of ACS (Amersham Corp.) and were counted by using a Packard Tri-Carb liquid scintillation counter, Model 1500.

Analysis of Isolated Peptides. Samples of peptides for amino acid analysis were hydrolyzed at 110 °C in the gas phase of 6 N HCl containing 1% phenol (after flushing with nitrogen) for 22 h in sealed, evacuated tubes. The hydrolysate was dried, converted into PTC amino acids, and analyzed with the PICO Tag amino acid analysis system (Waters).

Automated sequence analysis was performed on an Applied Biosystems gas-phase sequencer, Model 470A, equipped with an online PTH analyzer, Model 120, and computer, Model 900A. Typically, 200–1000 pmol of peptide was used for each analysis.

Isolation of Isocitrate Dehydrogenase Subunits by Chromatofocusing. A 3-mL column of PBE-94 resin was equilibrated with 0.02 M Tris–acetate buffer, pH 8.0, containing 6 M urea and 0.1 mM DTT. The enzyme sample (1 mL of 5 mg/mL) was applied in 50 mM Mes buffer, pH 7.0, containing 6 M urea and 0.1 mM DTT. Elution was performed with a buffer containing 3.6% (v/v) Polybuffer 96 and 8.4% (v/v) Polybuffer 74 (Pharmacia). The elution buffer also contained 6 M urea and 0.1 mM DTT and was titrated to pH 5.0 with acetic acid. The column was washed with 1 N NaCl between experiments. Fractions of 0.4 mL were collected; the pH was determined, and the protein concentration was measured by a dye binding method (Bio-Rad) based on the method of Bradford (1976). The identification of individual subunits was based upon comparison with the pattern obtained upon isoelectric focusing in polyacrylamide gels of unmodified enzyme in the presence of 6 M urea (Ramachandran & Colman, 1980). Gel rods were stained with Coomassie Blue.

The isolated subunits were adjusted to pH 8.0 and carboxymethylated as described above by using [1-^{14}C]iodoacetate. They were then dialyzed for 2 days against 4 L of 0.5 M ammonium bicarbonate (with two changes) to remove ampholytes, followed by dialysis against 4 L of 50 mM ammonium bicarbonate (with two changes). The individual subunits were digested with trypsin as described for the complete enzyme.

Results

Fractionation of Radioactive BrKG-Modified Peptides by HPLC. Pig heart NAD+-dependent isocitrate dehydrogenase was inactivated 80% by incubation for 150 min with 5 mM 3-bromo-2-ketoglutarate at pH 6.1. Enzyme reacted under similar conditions with 3-bromo-2-[^{14}C]ketoglutarate has been shown to incorporate 0.7 mol of reagent/mol of average subunit (Bednar et al., 1982a). After unreacted BrKG was removed, the carbonyl group of enzyme-bound reagent was reduced with [^{3}H]$NaBH_4$, yielding enzyme labeled with tritium at the reacted amino acids. The modified isocitrate dehydrogenase was carboxymethylated, dialyzed against ammonium bicarbonate at pH 8.0, treated with trypsin, and then lyophilized. The resultant digest, which contained 0.79 mol of ^{3}H/mol of average subunit, was examined by HPLC in 0.1% trifluoroacetic acid. Figure 1A,B illustrates the pattern of A_{220nm} and distribution of radioactivity in peptides derived from this inactive modified enzyme. The radioactivity of peak I was always small but present in variable amounts in different samples and was volatile upon lyophilization of the fraction; therefore, we consider that this radioactivity is not covalently bound to a peptide. The major labeled reagent peak is that designated peak V, which represents more than 50% of the radioactivity of peptide-associated tritium (peaks II–V) and coincides with an A_{220nm} peak centered at fraction 102, which is absent in unmodified enzyme. Control enzyme that was incubated in the absence of BrKG, treated with [^{3}H]$NaBH_4$, digested with trypsin, and lyophilized exhibited 0.40 mol of ^{3}H/mol of average subunit. This result suggests that the inactivated enzyme contained 0.39 mol of ^{3}H/mol of average subunit (0.79–0.40) associated with the carbonyl group of the enzyme-bound reagent. On HPLC, the distribution of radioactivity in peptides derived from this control enzyme exhibited only one peak, corresponding to peak I, and the low amount of radioactivity observed between the numbered peaks in Figure 1B. (The net radioactivity shown in Figure 1 has been corrected for background, but not for the radioactivity distributed in the digest of control enzyme.)

Enzyme incubated with BrKG in the presence of 10 mM isocitrate and 2 mM $MnSO_4$ for the same time period retains all of its activity, but still has 0.50 mol of ^{3}H/mol of average subunit (or 0.10 mol of ^{3}H/mol of average subunit if corrected for the radioactivity found in the control enzyme). Figure 1C shows the distribution of radioactivity in the tryptic digest peaks of this "protected enzyme". Peaks I and II are similar in size in the digest from the inactivated (Figure 1B) and protected (Figure 1C) enzymes. While peaks III and IV are decreased, the largest reduction in radioactivity in the protected enzyme is observed for peak V. Since modification of peak V by BrKG seems best to correlate with inactivation of isocitrate dehydrogenase, we have focused on the further purification of the labeled peptide from this radioactive peak contained in fractions 102–104 and eluting around 22.5% acetonitrile.

Figure 2A shows the result of subjecting peak V to HPLC at pH 5.8. The radioactive BrKG-modified peak occurs at 13.5% acetonitrile (i.e., 27% solvent D), separated from other unlabeled peptides. This peak contains a single peptide, as indicated by the unique amino acid sequence obtained on the gas-phase sequencer and recorded in Table I, column 1. The peptide applied to the sequencer contained 600 pmol of tritium, which is less than the average amount of amino acids (815 pmol) released in cycles 4–6. In any mixture of nonradioactive $NaBH_4$ and [^{3}H]$NaBH_4$, preferential reduction by $NaBH_4$ reflects a kinetic isotope effect. In this case, the kinetic isotope effect of the reaction is calculated as 1.36. This value is close to the kinetic isotope effect of 1.2–1.3 found for reduction of a bromodioxobutyl nucleotide covalently bound to pyruvate kinase (DeCamp & Colman, 1989) and only silghtly lower than that of 2.4 observed previously on reduction of 2-ketoglutarate bound to NADP-dependent isocitrate dehydrogenase (Ehrlich & Colman, 1987). Analysis of the modified peptide yielded the sequence

Ser-Ala-X-Val-Pro-Val-Asp-Phe-Glu-Glu-Val-Val-Val-Ser-Ser-Asn-Ala

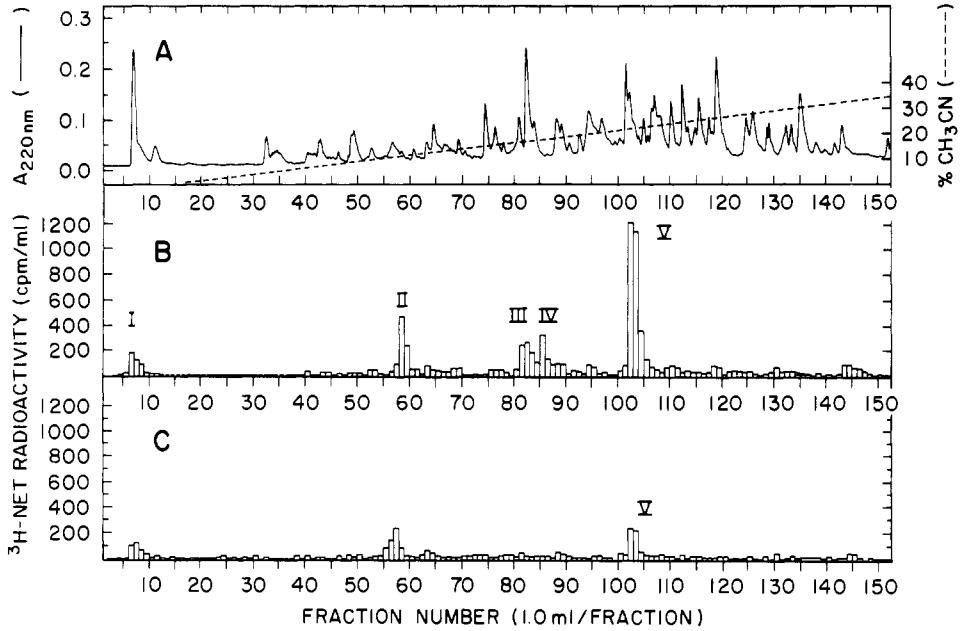

FIGURE 1: Fractionation of tryptic peptides from BrKG-modified isocitrate dehydrogenase by HPLC in 0.1% trifluoroacetic acid. Enzyme (2 mg) incubated with 5 mM 3-bromo-2-ketoglutarate for 150 min, either in the absence of ligands (A, B) or in the presence of 10 mM isocitrate and 2 mM $MnSO_4$ (C), was reduced with sodium [^{3}H]borohydride, carboxymethylated with nonradioactive iodoacetate, and digested with trypsin, as described under Experimental Procedures. Peptides were separated on a Vydac C-18 column equilibrated with 0.1% trifluoroacetic acid and using solvent system I as described under Experimental Procedures. (A) Absorbance at 220 nm. (B) Radioactivity, after background was subtracted, for tryptic digest of inactive enzyme modified in the presence of ligands. The distribution of radioactivity in peptide peaks II–V was 15.5%, 14.5%, 12%, and 58%, respectively. (C) Net radioactivity for tryptic digest of active enzyme modified in the presence of the protectants isocitrate and $MnSO_4$. In the several samples injected onto this column, 93–100% of the applied radioactivity was recovered in the fractions.

Table I: Amino Acid Sequences of Peptides V and V_u from NAD^+–Isocitrate Dehydrogenase[a]

cycle	amino acid	peptide V isolated from BrKG-modified enzyme (^{12}C carboxymethylation), amount[b] (pmol)	peptide V_u isolated from unmodified enzyme: sample 1 (^{12}C carboxymethylation), amount (pmol)	peptide V_u isolated from unmodified enzyme: sample 2 (^{14}C carboxymethylation), amount (pmol)	peptide V_u isolated from unmodified enzyme: sample 2 (^{14}C carboxymethylation), cpm[e]
1	Ser	184	162	96	2
2	Ala	1300	442	380	5
3	CmCys	X	405	214	454
4	Val	728	317	249	117
5	Pro	931	358	165	31
6	Val	786	365	231	9
7	Asp	338	194	106	4
8	Phe	779	384	186	4
9	Glu	256	116	162	3
10	Glu	332	305	199	4
11	Val	592	201	165	1
12	Val	194	61	54	0
13	Val	435	227	150	2
14	Ser	534	94	200	1
15	Ser	721	33	242	2
16	Asn	167	80	94	0
17	Ala	228[c]	121	117	5
18	Asp	*d*	42	42	0
19	Glu	*d*	46	51	0
20	Glu	*d*	71	35	0
21	Asp	*d*	41	35	1
22	Ile	*d*	16	42	0
23	Arg	*d*	18	18	0

[a] Amino acid sequences were determined for peptide V isolated from BrKG-modified enzyme as in Figure 2A and for peptide V_u purified from unmodified enzyme (sample 1 isolated as in Figure 2B; sample 2 isolated as in Figure 3B). [b] This peptide contained about 600 pmol of tritium on the basis of specific radioactivity per hydrogen in the original [^{3}H]$NaBH_4$ and the amount of radioactivity applied to the sequencer. [c] Not sequenced beyond cycle 17. [d] Another sample of about 120 pmol of peptide V was sequenced for 23 cycles and gave the following amounts of amino acids in the later cycles: 18, Asp (45 pmol); 19, Glu (13 pmol); 20, Glu (39 pmol); 21, asp (28 pmol); 22, Ile (15 pmol); and 23, Arg (8 pmol). [e] This peptide contained 250 pmol of [^{14}C]carboxymethyl groups on the basis of specific radioactivity of the iodoacetate and the amount of radioactivity in cycles 3–5.

The X at position 3 indicates that no phenylthiohydantoin derivative was detected in this cycle. Radioactivity was not detected in the fractions recovered from the sequencer cycles, suggesting that although the Edman reaction occurred, the PTH derivative of the modified amino acid was insoluble. Since the amino acid sequence of the pig heart NAD^+-dependent isocitrate dehydrogenase is unknown, it is difficult to identify directly the type of amino acid modified by 3-

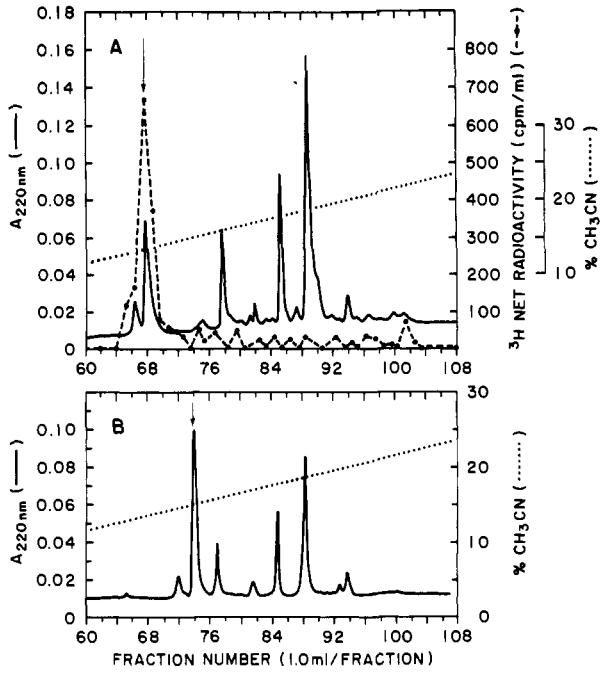

FIGURE 2: Further purification of peak V (or corresponding fractions from unmodified enzyme) by HPLC in 20 mM ammonium acetate, pH 5.8. (A) Radioactive peak V (fractions 102–104), obtained by HPLC of tryptic digest of BrKG-modified enzyme in 0.1% trifluoroacetic acid (Figure 1B), was subjected to HPLC on a Vydac C-18 column equilibrated with 20 mM ammonium acetate, pH 5.8, and using solvent system II as described under Experimental Procedures. (B) Corresponding fractions (102–104) obtained from HPLC in solvent system I of digest of enzyme not modified by BrKG. The arrows in (A) and (B) show, respectively, the elution positions of the modified (13.5% acetonitrile) and corresponding unmodified (15.0% acetonitrile) peptides. No other A_{220nm} or radioactive peaks were observed in regions of the chromatogram not shown.

bromo-2-ketoglutarate. Accordingly, we sought to identify the corresponding peptide from the proteolytic digest of control enzyme not modified by BrKG.

The tryptic hydrolysate of the unmodified enzyme was subjected to HPLC in 0.1% trifluoroacetic acid, and although the A_{220nm} pattern was somewhat different in the region of peak V, fractions 102–104 were pooled and further purified by HPLC at pH 5.8, with the result shown in Figure 2B. This chromatogram of the digest of unmodified enzyme lacks the peak centered at fraction 68 (13.5% acetonitrile) in Figure 2A, but it has a new peak (termed V_u) at fraction 74 (15.0% acetonitrile or 30% solvent D). The amino acid sequence of this peak was determined, as shown in Table I, column 2 (sample 1). This sample, which was sequenced to the C-terminal arginine of the tryptic peptide, yielded the primary structure

Ser-Ala-CmCys-Val-Pro-Val-Asp-Phe-Glu-Glu-Val-Val-
Val-Ser-Ser-Asn-Ala-Asp-Glu-Glu-Asp-Ile-Arg

The amino acid composition of this peptide, obtained after acid hydrolysis, is consistent with this sequence.[2] Of the 17 sequencer cycles shown for the modified peptide, 16 have the same amino acid as does the unmodified peptide. (Another sample of modified peptide V that was sequenced through 23 cycles gave the same sequence as unmodified peptide V_u, except for cycle 3.) (Carboxymethyl)cysteine appears at position 3 of the unmodified peptide, at which position no amino acid was identified for the modified peptide. This difference indicates that cysteine at position 3 of this peptide is the target of 3-bromo-2-ketoglutarate in NAD+-specific isocitrate dehydrogenase.

[2] These data were submitted to the scrutiny of the reviewers and will be furnished to the interested reader by writing directly to the authors.

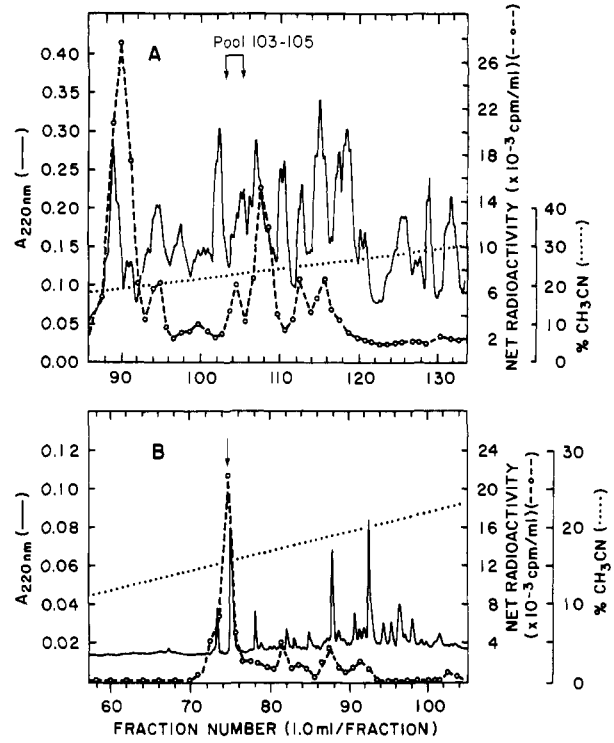

FIGURE 3: Purification of tryptic peptides from ^{14}C carboxymethylated complete enzyme not modified by BrKG. (A) HPLC in solvent system I of total tryptic digest. (B) Further fractionation in solvent system II of fractions 103–105 from (A). Only the relevant portion of the chromatogram is shown.

Separation by HPLC of [^{14}C](Carboxymethyl)cysteine Containing Peptides from Complete Isocitrate Dehydrogenase and Enzyme Subunits Not Modified by BrKG. Since the tryptic peptide V_u derived from unmodified enzyme contains a (carboxymethyl)cysteine residue, it is convenient to use [^{14}C]iodoacetate to introduce radioactivity into the (carboxymethyl)cysteine to serve as a tracer for this peptide in tryptic digests of complete unmodified isocitrate dehydrogenase and its separated subunits. To evaluate the feasibility of this approach, unmodified enzyme was carboxymethylated with [^{14}C]iodoacetate prior to incubation with trypsin. The digest was then purified first by HPLC in 0.1% trifluoroacetic acid (Figure 3A) and then by HPLC in ammonium acetate (Figure 3B). The difference in A_{220nm} patterns between unmodified and BrKG-modified enzyme digests can be observed by comparing Figures 3A and 1A in the region of fractions 100–106. The ^{14}C-labeled control enzyme had a radioactive peak centered at fraction 104, and when this peak was chromatographed at pH 5.8, it exhibited a ^{14}C peak centered at fraction 74 (Figure 3B). This peak yielded the same sequence as peptide V_u (Table I, sample 2), with the radioactivity appearing maximally in cycle 3 corresponding to [^{14}C](carboxymethyl)cysteine. In contrast to the results shown in Figure 3A, if enzyme was first modified with unlabeled BrKG and subsequently incubated with [^{14}C]iodoacetate, the resultant tryptic digest examined by HPLC was missing the radioactive peak centered at fraction 104 (Figure 3A); these observations indicate that prior reaction of isocitrate dehydrogenase with BrKG blocks the cysteine of peptide V from subsequent reaction with iodoacetate.

The α, β, and γ subunits of unmodified NAD+-dependent isocitrate dehydrogenase were separated by chromatofocusing

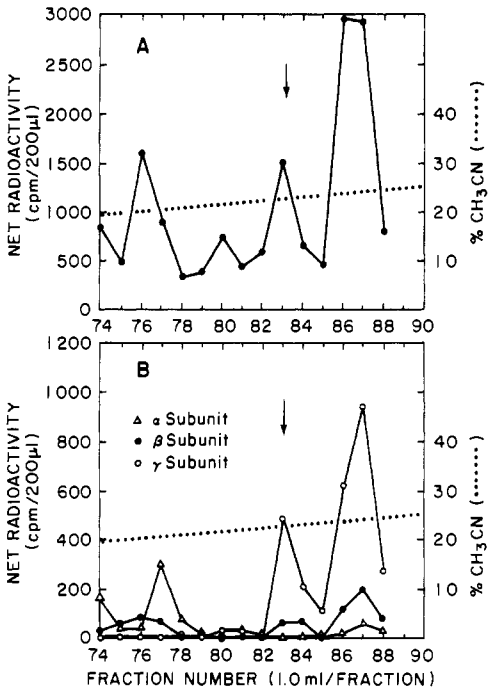

FIGURE 4: Fractionation by HPLC in 0.1% trifluoroacetic acid of tryptic peptides from ^{14}C carboxymethylated complete enzyme and separated subunits not modified by BrKG. The Vydac C-18 column, equilibrated with solvent A, was eluted for 10 min with the starting solvent. Thereafter, a linear gradient was run to 40% solvent B in 120 min followed by a linear gradient to 60% solvent B in the next 10 min. The flow rate was 1 mL/min. (This elution program is different from that termed solvent system I, as described under Experimental Procedures and used in Figure 3A.) (A) Radioactivity profile of tryptic digest of complete enzyme. (B) Radioactivity profile of tryptic digest of separate runs of α, β, and γ subunits. Only that region of the HPLC profile where peptide V_u elutes is shown, as indicated by the arrow.

in the presence of 6 M urea on a column of PBE-94 resin as described under Experimental Procedures, essentially as reported previously (Ehrlich & Colman 1983). On this column, the subunits are distinguished by the pH range of their elution: γ (7.4–6.7), β (6.3–6.0), and α (5.4–5.1), similar to earlier observations (Ramachandran & Colman, 1980; Ehrlich & Colman, 1983). The separated subunits were carboxymethylated with [1-^{14}C]iodoacetate, dialyzed to remove ampholytes, digested with trypsin, and fractionated by HPLC in 0.1% trifluoroacetic acid. The region of the chromatogram in which the unmodified peptide V_u elutes is compared in Figure 4 for complete enzyme (A) and the individual subunits (B). The arrow marks the elution position (at 22.5% acetonitrile) of peptide V_u in this solvent system. No radioactivity is observed in this region for α subunit and very little radioactivity for β subunit. A significant radioactive peak at 22.5% acetonitrile is noted only for complete enzyme and γ subunit. Each of these peaks was further purified, and the identification as peptide V_u was verified by gas-phase sequencing through the first five residues; the peak of radioactivity from (carboxymethyl)cysteine was found in cycle 3.

Chromatofocusing was also used to separate the subunits of complete enzyme that was first modified with BrKG and reduced with [^3H]$NaBH_4$. In this case, although the resolution of subunit types was not as good as in the case of unmodified enzyme, only fractions containing γ subunit yielded a radioactive peptide peak eluting at about 22.5% acetonitrile upon HPLC in 0.1% trifluoroacetic acid. After further purification by HPLC in ammonium acetate and gas-phase sequencing, the radioactive BrKG-modified peptide isolated from the γ subunit was confirmed to be that shown in Table I, column 1.

Discussion

3-Bromo-2-ketoglutarate functions as a classical affinity label of the substrate binding site of pig heart NAD^+-specific isocitrate dehydrogenase. It is structurally similar to isocitrate and to oxalosuccinate, the enzyme-bound intermediate of the enzymatic oxidative decarboxylation reaction. Although BrKG is a dicarboxylic acid, its electronegative bromine atom may occupy the site of the central carboxylate of the natural substrates of isocitrate dehydrogenase. The rate constant for inactivation exhibits a nonlinear dependence on BrKG concentration, consistent with reversible formation of an enzyme–reagent complex prior to irreversible reaction (Bednar et al., 1982a). Specific protection against inactivation is provided by isocitrate in the presence of Mn^{2+}, consistent with the requirement of divalent metal ion for tight binding of the substrate to the enzyme (Ehrlich & Colman, 1981). The total inactivation correlates with the incorporation of a limited amount of reagent incorporation. In the present paper, analysis of the location of reagent in the peptides derived from inactive enzyme suggests that the peptide contained in peak V is the predominant site of labeling, while lesser amounts of radioactivity are distributed among several other peptides. The major difference observed in comparing the labeled peptides derived from active enzyme incubated with BrKG in the presence of isocitrate and Mn^{2+} with those obtained from inactive enzyme is the large decrease in radioactive peak V. These results indicate that peak V contains a specific tryptic peptide labeled by 3-bromo-2-ketoglutarate that is at or near the metal–isocitrate site of NAD^+-specific isocitrate dehydrogenase.

After further purification of this critical peptide from BrKG-modified enzyme, gas-phase sequencing revealed a 23 amino acid peptide with C-terminal arginine, consistent with the specificity of trypsin. Although an unusual PTH derivative of an amino acid was not observed directly, no PTH amino acid was detected at cycle 3 from the BrKG-modified peptide. since a stoichiometric amount of (carboxymethyl)cysteine was present at position 3 in the corresponding peptide from enzyme not treated with BrKG, it is highly likely that a cysteine at position 3 is the target of 3-bromo-2-ketoglutarate. The BrKG-modified cysteine or its PTH derivative may either decompose under the acidic conditions encountered in the gas-phase sequencer or be insoluble in the solvents used so that it is not detected. The NAD^+-specific isocitrate dehydrogenase of pig heart has previously been reported to be inactivated by reaction at pH 6.0 of iodoacetate with 1–2 cysteines/average peptide chain (Mauck & Colman, 1976). In that case also, the inactivation rate was markedly decreased by the combined addition of Mn^{2+} and isocitrate, but not by NAD^+. Furthermore, the bovine heart NAD^+-dependent isocitrate dehydrogenase has also been shown to be inactivated by several sulfhydryl reagents, including 5,5′-dithiobis(2-nitrobenzoate), *p*-mercuribenzenesulfonate, and *N*-ethylmaleimide, with isocitrate and Mn^{2+} decreasing the inactivation rates (Fan & Plaut, 1974). However, in neither of these studies were the cysteine peptides identified. A critical cysteine of NAD^+-dependent isocitrate dehydrogenase, which is attacked by BrKG and has now been identified, was probably included among those modified in the earlier studies (Fan & Plaut, 1974; Mauck & Colman, 1976).

The nonallosteric $NADP^+$-specific isocitrate dehydrogenase of pig heart is also inactivated by covalent reaction with 3-bromo-2-ketoglutarate (Ehrlich & Colman, 1987). However,

BrKG causes only 65% inactivation of that enzyme as it reacts with but one of the enzyme's two identical subunits. Although BrKG binds reversibly to the $NADP^+$-dependent isocitrate dehydrogenase prior to irreversible inactivation (i.e., a plot of k_{obs} versus [BrKG] exhibits saturation kinetics), it does not appear to react at the same type of site in the two isocitrate dehydrogenases. Complete protection against inactivation of the nonallosteric enzyme is provided by $NADP^+$ or the $NADP^+$-α-ketoglutarate adduct rather than by manganous isocitrate, implying that BrKG modifies a residue in the nicotinamide region of the coenzyme site proximal to the substrate site. Furthermore, the 13 amino acid modified peptide isolated from the $NADP^+$-specific isocitrate dehydrogenase (Ehrlich & Colman, 1987) shows no resemblance to the peptide labeled by BrKG in the NAD^+-dependent enzyme. The two pig heart isocitrate dehydrogenases must have distinct active sites to react so differently with the same affinity label.

The allosteric NAD^+-specific isocitrate dehydrogenase of pig heart is composed of three nonidentical subunits present in the ratio 2α:1β:1γ (Ramachandran & Colman, 1980; Colman, 1983). The subunits have not yet been distinguished in their functions, but the use of site-specific affinity labels provides a possible approach to evaluating these functions. 3-Bromo-2-ketoglutarate, as an affinity label of the isocitrate site, binds in place of the substrate and can identify subunit participants in such sites provided that they have (in suitable proximity) an amino acid residue capable of reacting with the bromoketo group. The evidence presented in this paper indicates that a critical BrKG-modified peptide V comes from the γ subunit. The corresponding peptide V_u, derived from complete enzyme not incubated with BrKG, has a cysteine that is readily tagged with [^{14}C]iodoacetate, allowing identification of the subunit source(s) of the peptide. Comparison of the HPLC tryptic maps of the separated subunits reveals the γ subunit as the only major contributor of this cysteine peptide. Peptide V is clearly absent from α subunit, and the small amount of radioactivity in the region of peptide V from the β subunit sample (Figure 4B) may well be attributable to a minor level of γ subunit contamination of the β subunit fractions. Modification of the one γ subunit of the tetrameric protein appears to be sufficient to inactivate isocitrate dehydrogenase.

It has previously been demonstrated that native NAD^+-dependent isocitrate dehydrogenase binds tightly 2 mol of isocitrate/mol of tetrameric enzyme or 0.5 mol/mol of average subunit when Mn^{2+} is present (Ehrlich & Colman, 1981). Therefore, it is unlikely that the single γ subunit provides the only locus of isocitrate binding. It is possible that reaction of BrKG with the γ subunit directly blocks the binding of isocitrate on that subunit and has an indirect effect on another subunit, leading to decreased affinity for isocitrate or loss of catalytic activity. It must be pointed out, however, that in experiments in which complete enzyme was reversibly dissociated to subunits and the subunits were recombined in pairs both $\alpha\beta$ and $\alpha\gamma$ dimers exhibited appreciable ability to catalyze the oxidative decarboxylation of isocitrate (Ehrlich & Colman, 1983). Thus, in addition to an isocitrate site on the $\alpha\gamma$ dimer, the $\alpha\beta$ dimer must have at least one isocitrate binding site either solely within one of the subunit types or shared between the α and β subunits. The difference between the measured incorporation for 80% inactivation obtained previously by using 3-bromo-2-[^{14}C]ketoglutarate and that obtained in the present study by using [^{3}H]$NaBH_4$ may be relevant. Whereas the earlier value was 0.7 mol of reagent/mol of average subunit, the present net incorporation (after subtraction of the tritium measured in the control enzyme exposed to [^{3}H]$NaBH_4$ but not to BrKG) was only 0.39 mol of reagent/mol of average subunit. Even if corrected for an isotope effect of 1.36, the result is only 0.53 mol of reagent incorporated/mol of average subunit; since peak V accounts for 58% of the radioactivity found in inactive enzyme (Figure 1B), it corresponds to about 0.31 mol/mol of average subunit, a value consistent with its derivation from the γ subunit. The lower total incorporation measured in the present study may reflect loss of the reagent from subunit β or α prior to HPLC; this loss may be due to the instability (under the more drastic conditions used to isolate the protein digest in these experiments) of a different reaction product of BrKG with a corresponding residue on subunit β or α. Support for such a postulate must await the development of substrate site affinity labels with different types of reactive groups or the determination of the complete amino acid sequence of the several subunits of isocitrate dehydrogenase.

Registry No. BrKG, 76444-16-1; L-Cys, 52-90-4; isocitrate dehydrogenase, 9001-58-5.

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