Cysteinyl Peptides of Pig Heart NADP-dependent Isocitrate Dehydrogenase That Are Modified Upon Inactivation by \(N\)-Ethylmaleimide

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Pig heart NADP-specific isocitrate dehydrogenase is inactivated by \(N\)-ethylmaleimide (NEM) (Colman, R. F., and Chu, R. (1970) \textit{J. Biol. Chem.} 245, 601–607), and is completely protected against inactivation, but not against the incorporation of NEM, by isocitrate plus Mn\(^{2+}\). We have now treated the enzyme with \([\text{3H}]\)NEM in the absence and presence of isocitrate plus Mn\(^{2+}\), digested it with trypsin, and isolated and sequenced the labeled Cys peptides. In the inactive enzyme, two major peptides, SSGGFVWACK and DLAGCIHGLSNVK, and two minor peptides, CA-TITPDEAR and EPIICK, were labeled at Cys. Upon reaction with \([\text{3H}]\)NEM in the presence of isocitrate plus Mn\(^{2+}\), full catalytic activity was retained and only DLAGCIHGLSNVK was labeled; the Cys of this peptide is therefore not essential for catalysis. The modification of SSGGFVWACK appears to be the major cause of inactivation by NEM. The Cys in SSGGFV WACK may have a catalytic role, most likely in the strengthened binding of Mn\(^{2+}\) in the presence of isocitrate.

Isocitrate dehydrogenase was carboxymethylated under denaturing conditions with \(\text{[14C]}\)iodoacetate and digested with trypsin; 6 unique labeled Cys peptides, containing 6 unique Cys residues, were purified and sequenced. Six corresponding peptides were isolated from enzyme treated under denaturing conditions with \([\text{3H}]\)NEM. These results eliminate the previous uncertainty regarding the number of Cys residues in the enzyme. A comparison of the sequences of the \(\text{NH}_2\)-terminal 30 residues and the 6 Cys peptides of the pig heart NADP-dependent isocitrate dehydrogenase with the \textit{Escherichia coli} NADP enzyme provides evidence for great dissimilarity between the two enzymes.

Isocitrate dehydrogenase (isocitrate:NADP\(^+\) oxidoreductase (decarboxylating), EC 1.1.1.42) from pig heart mitochondria catalyzes the oxidative decarboxylation of isocitrate to \(\alpha\)-ketoglutarate. A divalent metal ion is required for activity (1), the highest activity being obtained with Mn\(^{2+}\) (2), and it is thought that the metal-isocitrate complex is the true substrate (3, 4). The enzyme is a dimer of identical subunits under many conditions (5, 6) and is not known to be allosterically regulated. Neither the sequence nor the three-dimen-

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\(^1\) The abbreviations used are: NEM, \(N\)-ethylmaleimide; HPLC, high performance liquid chromatography; PTH, phenylthiohydantoin; TPCK, \(N\)-tosylphenylalnine chloromethyl ketone.

\(^2\) Portions of this paper (including "Experimental Procedures" and "Results," Figs. 2-5, and Tables I-III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

EXPERIMENTAL PROCEDURES AND RESULTS

DISCUSSION

It is clear that, under denaturing conditions, 6 unique cysteine residues of pig heart NADP-dependent isocitrate dehydrogenase

The present study was undertaken to isolate and ascertain the amino acid sequence of tryptic peptides containing the cysteine residues of pig heart NADP-dependent isocitrate dehydrogenase that are modified when the enzyme reacts with NEM in the presence or in the absence of isocitrate and Mn\(^{2+}\), and hence the cysteine residue(s) that may be involved in the catalytic function of the enzyme. The total number of cysteine residues in the pig heart NADP-dependent isocitrate dehydrogenase has been reported to be 8.2–8.8 mol/mol subunit (15, 16), although the number of cysteines in isocitrate dehydrogenases from various sources ranges from 1 to 9 mol/mol subunit (4, 17, 18). We have now determined the total number of cysteine residues in the pig heart NADP-dependent isocitrate dehydrogenase by an independent method: the radioactive labeling, isolation, and sequencing, of all of the unique tryptic cysteinyl peptides. In addition, the sequence of the \(\text{NH}_2\)-terminal region of the protein has now been determined. A preliminary version of this work has been presented (19).
Cys Peptides of NADP Isocitrate Dehydrogenase

Fig. 1. Fractionation of tryptic digest of [14C]iodoacetate-labeled isocitrate dehydrogenase by HPLC in trifluoroacetic acid. Isocitrate dehydrogenase was treated with [14C]iodoacetate in 5 M guanidine hydrochloride and digested with trypsin as described under "Experimental Procedures." The trypsic digest was applied to a Vydac C₄₀ column equilibrated with 0.1% trifluoroacetic acid and eluted at a flow rate of 1 ml/min with Solvent System I. No significant absorbance at 220 nm or radioactivity was detected after 150 ml. A, absorbance of the eluate at 220 nm (—). Arrows, positions of the radioactive peaks. B, 14C net radioactivity per fraction (— — —).

In the region of Peptide IVbii, two proteolytic cleavages occurred that did not reflect the normal specificity of trypsin (Table I); both cleavages were incomplete. One of the cleavages occurred at a His-Ser bond, the other at a Tyr-Ala bond. Although no precedent was found for trypsic hydrolysis on the COOH-terminal side of His, there is precedent for tryptic hydrolysis on the COOH-terminal side of hydrophobic residues, even by trypsin preparations treated with TPCK (25). Such chymotryptic-like activity is thought to be due to ψ-trypsin, which arises by autolysis during prolonged incubation and an extra peak eluting at ~117 min. This extra peak was rechromatographed with Solvent System II. The resulting pure peptide, which yielded the sequence Asn-Ile-Leu-Gly-Thr-Val-Phe-Arg-Glu-Pro-Ile-Cys-Lys, features an internal Arg, and its COOH-terminal 6 residues correspond in sequence to Peptide I. The incomplete cleavage of the Arg-Glu bond under the milder digestion conditions accords with the known diminished rate of tryptic hydrolysis of peptide bonds close to an acidic side chain (25). Longer digestion periods with higher amounts of trypsin (5% for 6 h with a further 5% after 3 h) were therefore routinely used to ensure complete proteolysis. (The generation of Peptides I and VI also involves the cleavage of a peptide bond on the NH₂-terminal side of an acidic residue (Cys(Cm) and Asp, respectively), and the generation of Peptides II and V involves cleavage at a Lys preceded in the sequence by a Cys(Cm) residue. Yet all of these cleavages appear to have proceeded to completion even under the milder digestion conditions.)

1 In initial experiments, iodoacetate-labeled isocitrate dehydrogenase was digested for shorter time periods with lower amounts of trypsin (1% for 90 min with a further 1% added after 45 min). HPLC chromatograms of such tryptic digests displayed a diminished Peak II and an extra peak eluting at ~117 min. This extra peak was rechromatographed with Solvent System II. The resulting pure peptide, which yielded the sequence Asn-Ile-Leu-Gly-Thr-Val-Phe-Arg-Glu-Pro-Ile-Cys-Lys, features an internal Arg, and its COOH-terminal 6 residues correspond in sequence to Peptide I. The incomplete cleavage of the Arg-Glu bond under the milder digestion conditions accords with the known diminished rate of tryptic hydrolysis of peptide bonds close to an acidic side chain (25). Longer digestion periods with higher amounts of trypsin (5% for 6 h with a further 5% after 3 h) were therefore routinely used to ensure complete proteolysis. (The generation of Peptides I and VI also involves the cleavage of a peptide bond on the NH₂-terminal side of an acidic residue (Cys(Cm) and Asp, respectively), and the generation of Peptides II and V involves cleavage at a Lys preceded in the sequence by a Cys(Cm) residue. Yet all of these cleavages appear to have proceeded to completion even under the milder digestion conditions.)

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of trypsin and may be present in commercial preparations (26, 27). Specifically, the selective tryptic hydrolysis of a single Tyr-Ala bond in α-chymotrypsin has been previously reported (28). It is unlikely that such a cleavage in isocitrate dehydrogenase was catalyzed by contaminating chymotrypsin because the trypsin preparation had been treated with TPCK; furthermore, three other potential chymotryptic cleavage sites (Phe-Gln in Peptide IV and Phe-Val and Trp-Ala in Peptide V) remained intact even under our severe digestion conditions.

The use of [3H]NEM as an alternative to [14C]iodoacetate for labeling cysteine residues in isocitrate dehydrogenase yielded 6 major tryptic cysteinyl peptides (Fig. 5; Tables II and III) that correspond to the 6 [14C]iodoacetate-labeled cysteiny peptides (Fig. 1; Table I). The sequences of these 6 tryptic cysteinyl peptides are summarized in Table IV. NEM-labeled enzyme (Fig. 5) exhibited a single radioactive peak (IV), corresponding to Peaks IVa and IVb of iodoacetate-labeled enzyme (Fig. 1), indicating that the two unexpected tryptic cleavages in the region of Peptide IVbii, which were incomplete in iodoacetate-labeled enzyme, had proceeded to completion in NEM-labeled enzyme.

The labeling of only 6 unique tryptic cysteinyl peptides by two reagents that differ in their mechanism of reaction with thiols casts doubt on the previous quantitative determinations of the number of cysteine residues in the enzyme of 8.2–8.8 mol/mol subunit (15, 16). A possible source of error is the relative molecular mass of the enzyme subunit. The value used by Johanson and Colman (15, 16), 58,000, had been determined by sedimentation-equilibrium measurements on the native enzyme (8). However, this value is at the upper end of a range of values determined using different methods, including sedimentation equilibrium under denaturing conditions (52,900) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (48,500) (20). The use of the value of 48,500 instead of 58,000 yields a range of 6.9–7.4 mol/mol subunit from the data of Johanson and Colman (15, 16), considerably reducing the discrepancy.

A comparison of the labeled tryptic cysteinyl peptides from native isocitrate dehydrogenase treated with [3H]NEM in the absence (Fig. 4, A and B) or in the presence (Fig. 4, C and D) of isocitrate and Mn²⁺ shows that the cysteine residue in Peptide VI is not essential for activity: isocitrate plus Mn²⁺ protected the enzyme against inactivation by NEM but did not protect the cysteine of Peptide VI against modification by NEM. In the absence of isocitrate and Mn²⁺, NEM inactivated the enzyme and extensively modified not only Peptide VI, but also Peptide V, and partially modified Peptides I and II. The cysteines in Peptides I, II, and V are all protected by isocitrate plus Mn²⁺ against modification by NEM. In view of the extent of its reaction with NEM, the cysteine in Peptide V is likely to be the one whose modification causes the enzyme to lose activity. However, our results do not exclude the possibility that the modification of Peptides I or II also leads to inactive enzyme; indeed it is possible that all 3 cysteine residues, which protection studies suggest are at or near the substrate site, could react with NEM in a mutually exclusive manner to yield 3 different modified inactive enzymes, of which the predominant one is that modified at Peptide V.

These studies significantly extend those of Colman and Chu (13, 14), which yielded evidence for 2 major NEM-reactive cysteine residues: in the absence of isocitrate and Mn²⁺, the modification by NEM of one of these residues (Cys-A) inactivated the enzyme, while the modification of the other (Cys-B) did not affect the activity; and in the presence of isocitrate plus Mn²⁺, Cys-A was protected against modification, whereas Cys-B was modified, yielding a fully active enzyme. In the earlier studies, Cys-A and Cys-B were not identified. We have now isolated cysteinyl peptides that correspond to these residues: Peptide V presumably contains the critical substrate-protectable Cys-A, and Peptide VI the non-essential Cys-B, which is modified in the absence or presence of the substrate. Cys-A was postulated to be involved in the strengthened binding of manganous ion in the presence of isocitrate (29), so that Peptide V is expected to be close to the manganous isocitrate site.

The incorporation of NEM into native isocitrate dehydrogenase was determined by Colman and Chu (14) to be 2 mol/mol subunit in the absence or in the presence of isocitrate and Mn²⁺. The corresponding values of incorporation into the native enzyme determined in the present study were significantly lower but, as expected, they differed depending on whether the reaction was carried out in the absence (0.75 mol of reagent/mol of subunit) or in the presence (0.35 mol of reagent/mol of subunit) of isocitrate and Mn²⁺. The difference between the earlier and the present values of incorporation may be accounted for by the method of removal of the excess reagent: in the earlier studies, excess reagent was removed under non-denaturing conditions, allowing the possibility of residual non-covalent binding, whereas in the present studies it was removed under denaturing conditions. The difference between the incorporation in the absence of isocitrate and Mn²⁺, 0.40 mol/mol subunit, presumably corresponds to the incorporation into the protectable cysteine residues, those in Peptides I, II, and V. This difference, which is close to 0.5 mol/mol subunit, is most easily interpreted as the reaction of NEM at only 1 subunit per dimer; reaction of NEM can occur with any of the 3 protectable cysteine residues, of which that in Peptide V is the most reactive.

The product of the reaction of NEM and Cys is S-(N-ethylsuccinimidyl)cysteine (30). This compound is rather stable in acid; only upon prolonged acid hydrolysis is it converted to ethylamine and S-(1,2-dicarboxyethyl)cysteine (31). Therefore the adduct of a cysteine residue and NEM is expected to be stable during Edman degradation of the peptide in the gas-phase sequenator. Accordingly, a PTH-derivative of this adduct was detected in the cycle corresponding to Cys; furthermore, this derivative, which eluted as a double peak between Pro and Met, occurred in the cycle in which the bulk of the recovered radioactivity was detected. Because the radioactiv-

### Table IV

**Summary of sequences of cysteine-containing tryptic peptides from pig heart NADP-dependent isocitrate dehydrogenase**

| Peptide | HPLC peak numbers | Sequence |
|---------|-------------------|----------|
| I       | I, I, III, IV     | Cys-Ala-Thr-Ile-Thr-Pro-Asp-Glu-Ala-Arg |
| II      | II, IV            | Glu-Pro-Ile-Ile-Cys-Lys |
| III     | III, III          | Val-Cys-Val-Glu-Thr-Val-Glu-Ser-Gly-Ala-Met-Thr-Lys |
| IV      | IVa, IVa, IVb, IVbii, IVbii, IVbiii | Ala-His-Ser-Cys-Phe-Gln-Tyr-Ala-Ile-Gln-Lys |
| V       | V, V              | Ser-Ser-Gly-Phe-Val-Trp-Ala-Cys-Lys |
| VI      | VI, VI            | Asp-Leu-Ala-Gly-Cys-Ile-His-Gly-Leu-Ser-Asn-Val-Lys |
ity of the [3H]NEM resides in the N-ethyl group, the recovery of radioactivity indicates that the adduct had not hydrolyzed. Although \( S-(N\text{-ethy1succinimido}) \)cysteine could undergo partial hydrolysis to yield \( S-(1\text{-carboxy-2-N-ethylcarbamoy1}) \)ethylcysteine, the hydrolysis product is thought to recylize rapidly in acidic solution (31).

The reaction of Cys and NEM generates a new asymmetric center, giving rise to a pair of diastereomers (derivatives of a cysteiny1 peptide or of PTH-Cys). The diastereomeric adducts of NEM and Cys, which are interconvertible in acidic solution, have been resolved by ion-exchange chromatography (31). Such diastereomeric products could account for the double peaks observed both upon HPLC of NEM-labeled Peptides II, IV, and VI, in Solvent System II and upon HPLC of the PTH-Cys derivative resulting from Edman degradation. Fast atom bombardment-mass spectrometry of Peptide II, yielded an \( M \), for the \( m+1 \) parent ion consistent with the expected \( S-(N\text{-ethy1succinimido}) \)cysteine residue; and the observation of identical \( M \), values for both peaks of the \( A_{220} \) doublet rules out the possibility that one of the peaks corresponds to a partial hydrolysis product containing an \( S-(1\text{-carboxy-2-N-ethylcarbamoy1}) \)ethylcysteine residue, which can form under mildly alkaline conditions (31). This result agrees with the findings of Colman and Chu (14) who used paper chromatography to identify \( S-(N\text{-ethy1succinimido}) \)cysteine as the modified residue in NEM-treated isocitrate dehydrogenase.

\( S-(N\text{-ethy1succinimido}) \)cysteine is reported to undergo an intramolecular transamination reaction under very mildly alkaline conditions. This rearrangement, which proceeds to completion at \( pH \) \( 9.0 \), yields 2-(N-ethylcarbamoylmethyl)-3-keto-1,4-thiazine-5-carboxylic acid (30). Such a rearrangement could occur in the NH\(_2\)-terminal \( S-(N\text{-ethy1succinimido}) \)cysteine residue of Peptide I, during tryptic digestion at \( pH \) \( 8.0 \); it would block the NH\(_2\)-terminal amino group of Peptide I, and account for the absence of PTH-derivatives upon application of the peptide to the gas-phase sequenator. We therefore also expect the radioactive fragment generated by thermolytic cleavage of Peptide I, to be refractory to NH\(_2\)-terminal sequencing. The possibility of a blocked NH\(_2\) terminus is not excluded by fast atom bombardment mass spectrometry of Peptide I, which yielded an \( M \), for the \( m+1 \) parent ion consistent with both the \( S-(N\text{-ethy1succinimido}) \)cysteinyl peptide and its rearrangement product.

NADP-dependent isocitrate dehydrogenase from pig heart mitochondria is considered, on the basis of physical evidence, to consist of a single type of polypeptide chain (20). Our identification in this study of a unique NH\(_2\)-terminal sequence of 30 residues, combined with our isolation of 6 unique tryptic cysteiny1 peptides in approximately equal amounts, provides confirmatory evidence that the enzyme is indeed composed of a single type of subunit. The amino-terminal 30 residues of the pig heart enzyme show striking sequence similarity to the amino-terminal region of the NADP-dependent enzyme from yeast mitochondria (32), 20 out of 29 aligned residues being identical. In contrast, the amino-terminal region of the pig heart enzyme shows no apparent resemblance to the amino-terminal region of the Escherichia coli enzyme (33); nor do the cysteiny1 peptides of the pig heart enzyme show obvious similarity to any sequences of the E. coli enzyme.

In this study we have isolated and sequenced all of the tryptic cysteiny1 peptides of the pig heart mitochondrial NADP-dependent isocitrate dehydrogenase. The peptides were labeled using either of two thiol reagents, iodoacetate or NEM, that differ in the mechanism of their reaction with thiol groups. With both reagents, 6 unique cysteine residues were identified, yielding a definitive determination of the number of cysteine residues in isocitrate dehydrogenase.

NEM reacts rapidly with a cysteine residue in Peptide VI with no effect on activity, so that this cysteine is not essential for catalytic activity. NEM also reacts rapidly with 1 cysteine residue (in Peptide V) and slowly with 2 other cysteines (in Peptides I and II) concomitant with the inactivation of the enzyme. One or more of these 3 cysteines (which are probably at the substrate site) could be involved in catalysis, most likely by strengthening the binding of manganous ion in the presence of isocitrate.

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After elution with Solvent c, whether modified by treatment of the enzyme under denaturing conditions with [(3H)NEM] and reacted with [3H]Iodoacetate in acetonitrile) in 140 ml, then to guanidine hydrochloride in Buffer II (8 M, pH 7.7, containing 0.3 M MgCl$_2$, 1 M Tris[hydroxyethyl]ammonium chloride, pH 8.3, containing 10% glycerol and 0.2 M Na$_2$HPO$_4$). Fractions with specific activities greater than 25 unit/mg were pooled for further study; other fractions, which contained impurities, were discarded for rechromatography of the same column. The purified enzyme was confirmed by electrophoresis on a 0.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate at pH 8.3, using a silver stain. The specific activity of purified Cysteinyl peptide degradation was 25 unit/mg. Enzyme activity was determined from the recovery of di+/;? dihydroxyacetone (1 mol) in 30 min, as measured by monitoring the reduction of H$_2$O at 340 nm by the method of Bacon et al. (21). The resulting radioactive peptides were subjected to electrophoresis on 5% polyacrylamide gels containing 0.5% sodium dodecyl sulfate as described by Colman et al. (22). The fractions yielding primarily a single protein band (50% pure) were pooled. The concentrated enzyme (5 ml; 0.45-μm membrane filters) were lyophilized at 25°C, and lyophilized enzyme was dissolved in Buffer A (2.2 ml) anaerobically, containing 0.1% trifluoroacetic acid in Water (Solvent A). After elution for 10 min, the concentrated enzyme (5 ml; 0.45-μm membrane filters) were lyophilized at 25°C, and lyophilized enzyme was dissolved in Buffer A (2.2 ml) anaerobically, containing 0.1% trifluoroacetic acid in Water (Solvent A). After elution for 10 min, the enzyme solution was fractions eluted between Fractions 22 and 24. For the remainder of the isobelting experiments, multiple HPLC fractions were subjected to direct-insertion probe FAB mass spectrometry in order to determine the parent-totransition molecular masses. The complexes were run on a DFS 70/500 mass spectrometer equipped with a direct-insertion probe FAB mass spectrometer (Fisons Instruments). The matrix was glycerol containing -0.02 M HCl.

Preparation of Peptide Molecules from Isolated Cysteinyl Peptides Carboxymethylated with -(4)-Ethylmaleimide. Isolated dehydrogenase (2 mg) was dialyzed overnight (with one change of buffer) against 0.5% guanidine hydrochloride in Na$_2$SO$_4$·50 mM, pH 8.0. Diluted (final concentration, 0.15 M) was added and was added and was dialyzed for 3 hr at room temperature. The dialyzed solution (1 M, pH 8.3, containing 0.3 M MgCl$_2$, 1 M Tris[hydroxyethyl]ammonium chloride) was added to an additional 30 min, and the reaction was stopped by the addition of 2 M HCl. The enzyme solution was collected in 0.1 M, then dialyzed overnight at 4°C against 500 ml of 50 mM MgCl$_2$, pH 8.0, with two changes of buffer. The enzyme was then digested with trypsin (5% w/w) for 6 hr at 37°C, then to further 5% (w/w) of trypsin added after 3 h, and the digest was lyophilized.

Preparation of Peptide Molecules from Isolated Cysteinyl Peptides Carboxymethylated with -(4)-Ethylmaleimide. Isolated dehydrogenase (2 mg) was dialyzed overnight (with one change of buffer) against 0.5% guanidine hydrochloride in Na$_2$SO$_4$·50 mM, pH 8.0. Diluted (final concentration, 0.15 M) was added and was added and was dialyzed for 3 hr at room temperature. The dialyzed solution (1 M, pH 8.3, containing 0.3 M MgCl$_2$, 1 M Tris[hydroxyethyl]ammonium chloride) was added to an additional 30 min, and the reaction was stopped by the addition of 2 M HCl. The enzyme solution was collected in 0.1 M, then dialyzed overnight at 4°C against 500 ml of 50 mM MgCl$_2$, pH 8.0, with two changes of buffer. The enzyme was then digested with trypsin (5% w/w) for 6 hr at 37°C, then to further 5% (w/w) of trypsin added after 3 h, and the digest was lyophilized.

Determination of Incorporation of NEM Into Isolated Cysteinyl Peptides - Isolated dehydrogenase (2 mg) was reacted with [3H-NEM] as described above. At the desired time, the reaction was quenched by the addition of ethanol to a final concentration of 0.6 M. After 10 min, solid guanidine hydrochloride (0.8 mg/ml) was added in order to deurate the enzyme. The modified enzyme was separated from excess reagent by a column-chromatography procedure (23) involving two consecutive-S-500-ml Sephadex G-75 columns (0.5 ml of reaction mixture per column) equilibrated with 0.50 M Na$_2$SO$_4$, pH 6.0, containing 0.5 M guanidine hydrochloride. The average yield of protein after the two column-chromatography steps was 40%. The protein concentration in the eluate was determined by means of the Bio-Rad assay. As described in Experimental Procedures, the pooled lyophilized fractions from HPLC in trifluoroacetic acid (Fig. 1) were collected at 1 ml per column (1 M, pH 8.3, containing 0.3 M MgCl$_2$, 1 M Tris[hydroxyethyl]ammonium chloride) was added to an additional 30 min, then to further 5% (w/w) of trypsin added after 3 h, and the digest was lyophilized. The concentrated enzyme (5 ml; 0.45-μm membrane filters) were lyophilized at 25°C, and lyophilized enzyme was dissolved in Buffer A (2.2 ml) anaerobically, containing 0.1% trifluoroacetic acid in Water (Solvent A). After elution for 10 min, the enzyme solution was fractions eluted between Fractions 22 and 24. For the remainder of the isobelting experiments, multiple HPLC fractions were subjected to direct-insertion probe FAB mass spectrometry in order to determine the parent-totransition molecular masses. The complexes were run on a DFS 70/500 mass spectrometer equipped with a direct-insertion probe FAB mass spectrometry (Fisons Instruments). The matrix was glycerol containing -0.02 M HCl.

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The radioactive peptides were subjected to extended Edman degradation (Table 1). All of the peptides yielded FMOC-Gly in the cycle in which the bulk of the recovered radioactivity (80-90%) was detected. Peptide I had Arg as the C-terminal amino acid, and Peptides II, III, IVa, IVb, V, and VI have Lys as the C-terminal amino acid, consistent with the cleavage specificity of trypsin. However, Peptides IVA and VI had Tyr at the C-terminus. Inspection of Table 1 revealed a relationship among the four radioactive peaks derived from Peptides IVA and VI and the three radioactive peaks that were pooled in the region of Solvent System 3. These three radioactive peaks correspond to the three subsequences found (Peptides IVA, IVaii, and VI). Two of these tryptic carboxymethylcysteinyl peptides correspond to the three subsequences found (Peptides IVA, IVaii, and VI). The C-terminal sequence of each of these tryptic carboxymethylcysteinyl peptides is consistent with the cleavage specificity of trypsin. This is indicated by the diminished radioactivity that had not been treated with NEM. Radioactive peaks found upon chromatography of Peptide IVA, in the cycle in which the bulk of the recovered radioactivity (80-90%) was detected, were subjected to automated Edman degradation (Table 1). The amino acid sequence of the peaks is consistent with the cleavage specificity of trypsin, that is, the C-terminal amino acid, as indicated in Table 1.

**Table 1.**

| Peptide | C-terminal Residue | Radioactivity (%) |
|---------|--------------------|------------------|
| IVA     | Lys                 | 80-90            |
| IVaii   | Lys                 | 80-90            |
| VI      | Lys                 | 80-90            |
| IVA     | Lys                 | 80-90            |
| IVaii   | Lys                 | 80-90            |
| VI      | Lys                 | 80-90            |

**Fig. 2—continued**

**Fig. 2.** Fractionation of tryptic digest of isocitrate dehydrogenase labeled with [3H]-isocitrate or [14C]-isocitrate after prior modification with NEM in the absence or presence of isocitrate and MnCl₂. Isocitrate dehydrogenase (1.8 mg) in buffer D was treated with NEM (10 μM) for 10 min at 30°C either in the absence or in the presence of 3 mM L-isocitrate and 2 mM MgCl₂, or was left untreated. Each sample of enzyme was carboxymethylated with iodo[14C]-acetic acid, digested with trypsin, and chromatographed on a Zorbax C8 HPLC column with a solvent system I as described Experimental Procedures. **A.** Enzyme not modified by NEM. **B.** Enzyme modified by NEM, with no substrate added. **C.** Enzyme modified by NEM in the presence of isocitrate plus M [fully active]. **D.** Enzyme modified by NEM in the presence of isocitrate plus M [fully active], but also in the absence of isocitrate. **E.** Enzyme modified by NEM in the presence of isocitrate plus M [fully active], but also in the absence of isocitrate and MnCl₂. **F.** Enzyme modified by NEM in the presence of isocitrate plus M [fully active], but also in the absence of isocitrate and MnCl₂. **G.** Enzyme modified by NEM in the presence of isocitrate plus M [fully active], but also in the absence of isocitrate and MnCl₂.
The chromatogram for unmodified enzyme (Fig. 4A, B) displays the five major peaks of radioactivity (I, II, III, IV, and V) previously observed (Fig. 1). On the chromatogram for unmodified substrate (Fig. 4C, D) the peaks are strikingly decreased (c.f. Fig. 3A), indicating that the cysteine residue in each of the corresponding peptides had largely reacted with NEM. By contrast, on the chromatogram for protected substrate (Fig. 4B), only peak VI is decreased. Thus, in the presence of isocitrate plus Mn2+ Peptide V is essentially fully protected against modification by NEM whereas Peptide VI reacted with NEM in the absence of isocitrate and Mn2+.

Identification of Cysteine-Derived Peptides That React with NEM - With the goal of directly identifying by sequence analysis the cysteine residues of isocitrate dehydrogenase that react with NEM, we treated enzyme with [15NH4]HCl to deacetylate the enzyme followed by [15NH4]HCl to deacylate the enzyme. Thus, only those peptide segments that are identical to those of native isocitrate dehydrogenase were identified. The radioactivity was accompanied by a 5 kDa radioactive band on the HPLC column. To identify the radioactivity of this band, we subjected the radioactive peaks to the gas-phase sequencer (Table 1, Column 12) except for the derivative at Position 6 which was not sequenced.

Table 1.

| Table 1. Peptide Sequences of NEM-Labeled Isocitrate Dehydrogenase |  |  |
|-------------------------------------------------|---|---|
| Peptide | NEM-Labeled Peptide | Protected Peptide |
| Peptide | Sequence | Sequence |
| Peptide | (Table 1) | (Table 1) |
| Peptide | (1, 2, 3, 4) | (1, 2, 3, 4) |
| Peptide | (5, 6, 7) | (5, 6, 7) |
| Peptide | (8, 9, 10) | (8, 9, 10) |
| Peptide | (11, 12) | (11, 12) |
| Peptide | (13, 14) | (13, 14) |

We considered the possibility that, in enzyme essentially inactivated, the pattern of labeled peptides might be simpler than that observed for NEM inactivated enzyme (Fig. 4A, B). Accordingly, enzyme was incubated with [15NH4]HCl to deacylate the enzyme for 60 min in the absence of isocitrate and Mn2+. After partial acetylation of the enzyme, the peak at the major radioactive peak was identified by sequence analysis (Table 1, Column 12) except for the derivative at Position 6 which was not sequenced. The sequence analysis of the radioactive peak at the major radioactive peak was identical to that of Peptide V (Table 1, Column 12) except for the derivative at Position 6 which was not sequenced. The sequence analysis of the radioactive peak at the major radioactive peak was identical to that of Peptide V (Table 1, Column 12) except for the derivative at Position 6 which was not sequenced.

In conclusion, the presence of [15NH4]HCl was not sufficient to protect the enzyme against modification by NEM. Thus, the major radioactive peak at the major radioactive peak was identified by sequence analysis (Table 1, Column 12) except for the derivative at Position 6 which was not sequenced. The sequence analysis of the radioactive peak at the major radioactive peak was identical to that of Peptide V (Table 1, Column 12) except for the derivative at Position 6 which was not sequenced. The sequence analysis of the radioactive peak at the major radioactive peak was identical to that of Peptide V (Table 1, Column 12) except for the derivative at Position 6 which was not sequenced.

The chromatograms on the HPLC columns were monitored at 220 nm. The absorbance at 220 nm was essentially identical to that of Peptide V 

The chromatogram for unmodified enzyme (Fig. 4A, 4B) displays the five major peaks of radioactivity (I, II, III, IV, and V) previously observed (Fig. 1). On the chromatogram for unmodified enzyme (Fig. 4A, 4B) the peaks are strikingly decreased (c.f. Fig. 3A), indicating that the cysteine residue in each of the corresponding peptides had largely reacted with NEM. By contrast, on the chromatogram for protected enzyme (Fig. 4B), only peak VI is decreased. Thus, in the presence of isocitrate plus Mn2+ Peptide V is essentially fully protected against modification by NEM whereas Peptide VI reacted with NEM in the absence of isocitrate and Mn2+.

Identification of Cysteine-Derived Peptides That React with NEM - With the goal of directly identifying by sequence analysis the cysteine residues of isocitrate dehydrogenase that react with NEM, we treated enzyme with [15NH4]HCl to deacetylate the enzyme followed by [15NH4]HCl to deacylate the enzyme. Thus, only those peptide segments that are identical to those of native isocitrate dehydrogenase were identified. The radioactivity was accompanied by a 5 kDa radioactive band on the HPLC column. To identify the radioactivity of this band, we subjected the radioactive peaks to the gas-phase sequencer (Table 1, Column 12) except for the derivative at Position 6 which was not sequenced.

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| Peptide | (Table 1) | (Table 1) |
| Peptide | (1, 2, 3, 4) | (1, 2, 3, 4) |
| Peptide | (5, 6, 7) | (5, 6, 7) |
| Peptide | (8, 9, 10) | (8, 9, 10) |
| Peptide | (11, 12) | (11, 12) |
| Peptide | (13, 14) | (13, 14) |

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Table III

| Peptide | Alanine sequence of [3H]NEM-labeled cysteine peptides not listed in Table II |
|---------|------------------------------------------------------------------------------|

| Cycle number | Alanine (amino acid) | Alanine (amino acid) | Alanine (amino acid) |
|--------------|----------------------|----------------------|----------------------|
| 1            | Ile (420)            | Val (170)            | Ser (267)            |
| 2            | Thr (450)            | X                    | Z                    |
| 3            | Pro (512)            | Val (110)            | Phe (760)            |
| 4            | Asp (376)            | Glu (827)            | Gln (760)            |
| 5            | Glu (625)            | Thr (488)            | Tyr (206)            |
| 6            | Ala (696)            | Val (694)            |                     |
| 7            | Arg (450)            | Arg (209)            |                     |
| 8            | Gly (410)            | Glu (618)            |                     |
| 9            | Asp (494)            | Glu (618)            |                     |
| 10           | Ala (684)            | Glu (618)            |                     |
| 11           | Arg (209)            | Glu (618)            |                     |
| 12           | Thr (264)            |                     |                     |
| 13           | Lys (285)            |                     |                     |

A peptide in which the most radioactivity was detected.

(See also Footnote a, Table II.)

A mass spectrometry was used to obtain the parent-to-molecular mass of selected NEM peptides, primarily to determine whether their masses were consistent with the presence of the expected derivative of cystine. The mass spectrometer used was a VG ZAB-HS machine, which was operated in the selected ion-monitoring mode. The mass of each amino acid residue was determined to be within 0.5% of the expected value. The mass of each peptide was determined to be within 1% of the expected value. The mass of each peptide was determined to be within 1% of the expected value.

B-Terminal Sequencing Analysis - In order to compare to the pig heart NAD-dependent isocitrate dehydrogenase to other isocitrate dehydrogenases to which it might be related, the N-terminal sequence of NEM-labeled cysteine peptide corresponding to peptide I was determined by the method of Edman and Begg. The peptide was subjected to FAB mass characterization; the calculated value for peptide I was 1200.5: the observed value was 1200.5, which is identical to the calculated value. The calculated value for peptide II was 1201.7: the observed value was 1201.7, which is identical to the calculated value. The calculated value for peptide III was 1201.7: the observed value was 1201.7, which is identical to the calculated value. The calculated value for peptide IV was 1201.7: the observed value was 1201.7, which is identical to the calculated value. The calculated value for peptide V was 1201.7: the observed value was 1201.7, which is identical to the calculated value. The calculated value for peptide VI was 1201.7: the observed value was 1201.7, which is identical to the calculated value. The calculated value for peptide VII was 1201.7: the observed value was 1201.7, which is identical to the calculated value.

* the calculated value for peptide I was determined to be within 1% of the expected value. The calculated value for peptide II was determined to be within 1% of the expected value. The calculated value for peptide III was determined to be within 1% of the expected value. The calculated value for peptide IV was determined to be within 1% of the expected value. The calculated value for peptide V was determined to be within 1% of the expected value. The calculated value for peptide VI was determined to be within 1% of the expected value. The calculated value for peptide VII was determined to be within 1% of the expected value.