Subunit Location and Sequences of the Cysteinyl Peptides of Pig Heart NAD-Dependent Isocitrate Dehydrogenase†

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ABSTRACT: Pig heart NAD-dependent isocitrate dehydrogenase has a subunit structure consisting of $\alpha_2\beta\gamma$, with the α subunit exhibiting a molecular weight of 39 000 and the β and γ each having molecular weights of 41 000. The amino-terminal sequences (33–35 residues) and the cysteinyl peptide sequences have now been determined by using subunits separated by chromatofocusing or isoelectric focusing and electroblotting. Displacement of the N-terminal sequence of the α subunit by 11–12 amino acids relative to that of the larger β and γ subunits reveals a 17 amino acid region of great similarity in which 10 residues are identical in all three subunits. The complete enzyme has 6.0 free SH groups per average subunit of 40 000 daltons, but yields 15 distinguishable cysteines in isolated tryptic peptides. Six distinct cysteines in sequenced peptides have been located in the α subunit. The β and γ subunits contain seven and five cysteines, respectively, with tryptic peptides containing three cysteines being common to the β and γ subunits. The three subunits appear to be closely related, but β and γ are more similar to each other than either is to the α subunit. The NAD-specific isocitrate dehydrogenase from pig heart has been shown to have 2 binding sites/enzyme tetramer for isocitrate, manganous ion, NAD^+, and the allosteric activator ADP [Colman, R. F. (1983) *Pept. Protein Rev. 1*, 41–69]. It is proposed that the catalytically active tetrameric enzyme is organized as a dimer of dimers in which the $\alpha\beta$ and $\alpha\gamma$ dimers are nonidentical but functionally similar.

The pig heart NAD-dependent isocitrate dehydrogenase [isocitrate:NAD oxidoreductase (decarboxylating), EC 1.1.1.41] is an allosteric enzyme activated by ADP (Cohen, 1972; Colman, 1983). The enzyme is a tetramer of three distinguishable types of subunits in the approximate ratio $2\alpha:1\beta:1\gamma$ with similar molecular weights of 39 000 for the α subunit and of 41 000 for the β and γ subunits (Ramachandran & Colman, 1980). The minimum molecular weight is 160 000 for a complete enzyme tetramer (Ehrlich et al., 1981). The subunits are distinguished by their isoelectric points (5.6–5.8, 6.4–6.7, and 6.9–7.5, respectively, for the multiple bands of α, β, and γ subunits), and these can be separated by isoelectric focusing in polyacrylamide gels (Ramachandran & Colman, 1980). Alanine is the N-terminal amino acid of both the α and the β chains, whereas phenylalanine is the N-terminal of the γ subunit (Ramachandran & Colman, 1980). The individual bands within each region have been shown to have the same amino acid composition, whereas the amino acid composition and tryptic peptide map (as determined by paper chromatography and electrophoresis) of each subunit type were found to be different from the other two (Ramachandran & Colman, 1980). Measurements of the equilibrium binding of the ligands isocitrate, manganous ion, ADP, NAD^+, and NADH have revealed that the NAD-specific isocitrate dehydrogenase has 0.5 site/average subunit or 2 sites/enzyme tetramer for each ligand (Ehrlich & Colman, 1981, 1982). In view of the subunit diversity, the possibility must be considered that the subunits have specialized functions.

Several types of amino acid, including lysine, glutamate, aspartate, cysteine, and arginine, have been implicated by chemical modification of isocitrate dehydrogenase as being important for catalysis and regulation (Colman, 1983). Affinity labeling by substrate (Bednar et al., 1982a,b) and nucleotide analogues (King & Colman, 1983; Huang & Colman, 1984; Huang et al., 1986) has been used to probe specific sites. However, the complete amino acid sequence of the NAD-dependent isocitrate dehydrogenase is not yet known, and in only two cases have the chemically modified peptides been isolated and their sequences determined (Huang & Colman, 1989; Saha et al., 1989). Further characterization of the subunits is essential in order to understand the relationship between the enzyme structure and its catalytic and regulatory sites.

In this paper, we describe a new procedure for isolating the subunits of NAD-dependent isocitrate dehydrogenase by electroblotting which complements the previously reported method of chromatofocusing. We present and compare the amino-terminal sequences of the α, β, and γ subunits, as well as the sequences of the (carboxymethyl)cysteine-containing peptides from all the subunits. The results of the sequencing analyses elucidate the relationships among the enzyme's subunits.

Experimental Procedures

Materials. 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS),[1] *N*-tosylphenylalanine chloromethyl ketone treated trypsin, dithiothreitol (DTT), MES, and ammonium bicarbonate were supplied by Sigma. Ultrapure urea was purchased from Schwarz/Mann and [1-^{14}C]iodoacetic acid from New England Nuclear Corp. PBE-94 resin and buffers used for chromatofocusing were obtained from Pharmacia. Acrylamide, bis(acrylamide), ampholytes, and other chemicals required for the isoelectric focusing gels were supplied by Bio-Rad, and Immobilon P (PVDF) was supplied by Millipore. Methanol, acetonitrile, and trifluoroacetic acid were obtained from Fisher Co.

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[1] Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; DTT, dithiothreitol; MES, 2-(*N*-morpholino)ethanesulfonic acid; PVDB, poly(vinylidene difluoride) (Immobilon); TPCK, *N*-tosylphenylalanine chloromethyl ketone; CmCys, (carboxymethyl)cysteine.

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NAD-dependent isocitrate dehydrogenase was isolated and 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 7.0, containing 20% glycerol and 0.2 mM $MnSO_4$ and was stored at −75 °C. Enzyme concentrations in mg/mL were calculated by using the optical density at 280 nm multiplied by 1.55 (Shen et al., 1974).

Cysteine Determination. Aliquots of NAD-dependent isocitrate dehydrogenase (0.2 mL of 0.6 mg/mL in 50 mM MES buffer containing 20% glycerol) were added to 0.6 mL of 200 mM Tris-HCl buffer, pH 8.0, in a 1.0-mL cuvette. Sodium dodecyl sulfate (0.1 mL of a 10% w/w stock solution) was added in order to denature the protein. The absorbance of the solution was read at 412 nm against a protein-free buffer blank which was otherwise identical with the test solution. A freshly prepared solution of 10 mM 5,5′-dithiobis(2-nitrobenzoate) (0.1 mL) was added to each cuvette, and the concentration of free SH groups in the enzyme was calculated from the change in absorbance. A molar extinction coefficient of 13 600 M^{-1} cm^{-1} at 412 nm for thionitrobenzoate was utilized in the calculations (Ellman, 1958).

Preparation of Tryptic Peptides from Complete Enzyme Carboxymethylated with [1-^{14}C]Iodoacetate. Native NAD-dependent isocitrate dehydrogenase (2 mg) was dialyzed overnight at 4 °C against 50 mM ammonium bicarbonate, pH 8.0, containing 6 M urea and 0.1 mM DTT. An amount of DTT was added which was equal to 10 times the number of moles of cysteine present in the enzyme. After 1 h, [1-^{14}C]-iodoacetic acid (specific radioactivity 2.4×10^{12} cpm/mol) was added in an amount equal to 2.2 times the number of moles of DTT, followed 30 min later by the addition of excess β-mercaptoethanol to quench the reaction. The carboxymethylated enzyme (approximately 2 mL) was dialyzed at 4 °C against two changes of 50 mM ammonium bicarbonate, pH 8.0 (4 L), to remove excess reagents.

The ^{14}C-labeled carboxymethylated enzyme was then digested at 37 °C for 2 h in 50 mM ammonium bicarbonate (pH 8.0) with TPCK-treated trypsin, 1:20 (w/w) with respect to isocitrate dehydrogenase. Following the addition of a second identical aliquot of trypsin, the digestion was continued at 37 °C for another 2 h, after which the sample was lyophilized.

Isolation of Isocitrate Dehydrogenase Subunits. (A) By Chromatofocusing. The α, β, and γ subunits of NAD-dependent isocitrate dehydrogenase were separated by chromatofocusing using a 3-mL column of PBE-94 resin equilibrated with 0.02 M Tris acetate buffer, pH 8.0, containing 6 M urea and 0.1 mM DTT, as described previously (Huang & Colman, 1989). The identification of individual subunits was made by comparison with the pattern obtained upon isoelectric focusing in polyacrylamide gels of complete 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. Samples were then dialyzed for 2 days against 4 L of a 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.

(B) By Electroblotting. The NAD-dependent isocitrate dehydrogenase (4 mg) was dialyzed overnight at 4 °C against 50 mM ammonium bicarbonate, pH 8.0, containing 6 M urea. The enzyme was carboxymethylated with [1-^{14}C]iodoacetate as described above and was then dialyzed overnight at 4 °C against 50 mM MES buffer, pH 7.0, containing 20% glycerol, 6 M urea, and 0.2 mM $MnSO_4$ before being subjected to isoelectric focusing in a pH range from 5 to 8 in the presence of 6 M urea according to the procedure described by Ramachandran and Colman (1980), using a polyacrylamide plate gel (8 cm × 8 cm). One strip of the gel was sliced, stained, and destained (by the method outlined by Bio-Rad) as described by Hayman and Colman (1982). The rest of the gel was saved for electroblotting.

The unstained isoelectric focusing plate gel was soaked in 3.5% perchloric acid for 50 min (with five changes) and then equilibrated in 2% acetic acid for 20 min. The gel was transferred into a blotting cassette (Bio-Rad) and was subjected to electrophoresis at constant current (150 mA) in 1% acetic acid for 1.5 h to remove the ampholytes. The gel was then soaked in transferring buffer (10 mM CAPS, pH 11.0, containing 6 M urea and 0.1 mM DTT) for 20 min, after which it was inserted between the Immobilon P membrane (PVDF) and filter papers. The electroblotting was performed at constant current (250 mA) for 24 h at 4 °C with 60–70% yield. After transferring, one strip of Immobilon paper was cut and the subunit bands were visualized by staining the membrane with Coomassie Blue (1% Coomassie Blue in a solution of 50% methanol and 10% acetic acid). After destaining with 50% methanol and 10% acetic acid, the Immobilon paper was washed three times with HPLC-grade water and the sections containing the individual subunits were separated by cutting the unstained paper at the same distance from the origin as the stained bands.

The PVDF membranes containing individual subunits were incubated separately in 40% acetonitrile containing 0.05% trifluoroacetic acid at 50 °C for 45 min. After four consecutive extractions, the eluants from each subunit type were combined and lyophilized. About 75%, 94%, and 93%, respectively, of the total radioactivity in the α, β, and γ subunits was recovered. Each subunit was redissolved in 1 mL of 50 mM ammonium bicarbonate (pH 8.0) and digested with trypsin as described above.

Separation of Tryptic Peptides by High-Performance Liquid Chromatography. The tryptic peptides from ^{14}C-labeled carboxymethylated enzymes were separated on a Vydac C_{18} column (0.46 × 25 cm) using a Varian Model 5000 HPLC system equipped with a Varichrome absorbance monitor. Two different solvent systems were used. For *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 40% solvent B (acetonitrile containing 0.07% trifluoroacetic acid) in 120 min, followed by a linear gradient to 60% B in the next 10 min. For *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 (200 μL) of fractions were mixed with 5 mL of ACS (Amersham Corp.) and were counted by using a Packard Tri-Carb liquid scintillation counter, Model 1500.

Analysis of Isolated Peptides. 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 of peptide purified from

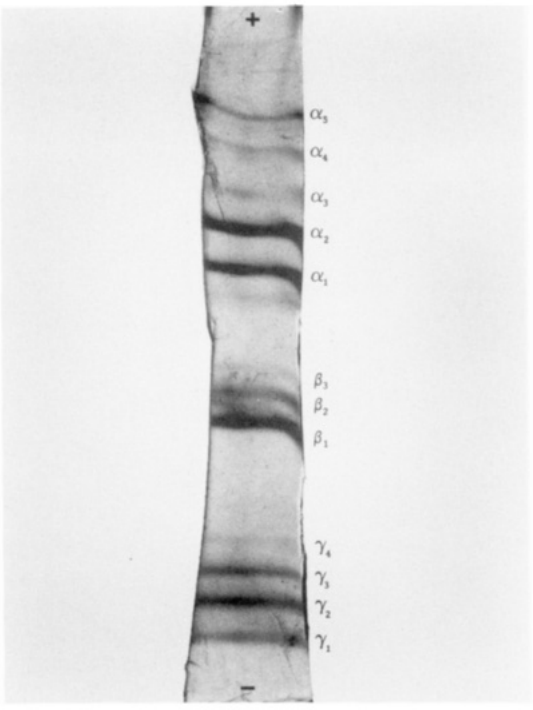

FIGURE 1: Isoelectric focusing in polyacrylamide gel plates. Isoelectric focusing was performed in a plate gel in the presence of 6 M urea from pH 5 to 8 as described in the text. One strip of gel was sliced and stained. The identification of α, β, and γ subunits is by comparison with the pattern obtained by Ramachandran and Colman (1980).

the HPLC system. The peptide bands from Immobilon P membrane, visualized by Coomassie Blue staining, were sequenced directly, band by band, using 20–60 pmol. The excised paper was washed three times with HPLC-grade water before sequencing.

RESULTS

Identification of Subunits by Isoelectric Focusing in Polyacrylamide Gel Plates and Amino-Terminal Sequence Determination. The pig heart NAD-dependent isocitrate dehydrogenase was subjected to analytical isoelectric focusing under denaturing conditions in polyacrylamide plate gels containing 6 M urea. A typical protein band pattern, shown in Figure 1, is similar to that reported earlier for this enzyme using tube gels (Ramachandran & Colman, 1980); these bands have been classified into an α group (isoelectric points 5.1–5.4), a β group (isoelectric points 6.0–6.3), and a γ group (isoelectric point 6.7–7.4) on the basis of similar amino acid compositions (Ramachandran & Colman, 1980). There are two major bands of α subunit, two for β subunit, and 3 for γ subunit.

After the subunit bands were transferred electrophoretically to Immobilon, the amino acid sequence of each was determined by applying the Immobilon strip directly to the gas-phase sequencer. Representative results are shown in Table I. For the first 25 residues, the amino acid sequences of α_1 and α_2 are identical; but only α_2 was sequenced as far as 35 residues: A-G-G-V-K-T-V-T-L-I-P-G-D-G-I-G-P-E-I-S-A-A-V-M-K-I-F-D-A-A-K-A-P-I-Q. Although present in lower amounts, the PTH-amino acids derived from α_3, α_4, and α_5 are also consistent with this sequence. The light band appearing just below α_1 (Figure 1) has the same sequence but could only be determined as far as 15 residues.

Automated Edman degradation of subunit bands β_1 and β_2 gave the same sequence: A-S-R-S-Q-A-E-D-V-R-V-E-G-A-F-P-V-T-M-L-P-G-D-G-V-G-P-E-L-M-A-A-V. Also consistent with this sequence, although detected in lower amounts, was subunit β_3.

Table I: Determination of Amino-Terminal Sequences of Individual Subunit Bands Obtained by Electrotransblotting

cycle	amino acid	subunit α_1 (pmol)	amino acid	subunit β_1 (pmol)	amino acid	subunit γ_1 (pmol)
1	Ala	94[a]	Ala	61[c]	Phe	21[d]
2	Gly	65	Ser	75	Ser	33
3	Gly	65	Arg	6	Gln	15
4	Val	70	Ser	49	Gln	16
5	Lys	190	Gln	32	Thr	20
6	Thr	113	Ala	39	Ile	41
7	Val	64	Glu	24	Pro	21
8	Thr	106	Asp	17	Pro	22
9	Leu	79	Val	29	Ser	16
10	Ile	64	Arg	10	Ala	16
11	Pro	58	Val	29	Lys	5
12	Glyu	70	Glu	22	Tyr	8
13	Asp	47	Gly	26	Gly	15
14	Gly	66	Ala	32	Gly	19
15	Ile	70	Phe	18	Ile	41
16	Gly	66	Pro	22	Leu	12
17	Pro	49	Val	23	Thr	13
18	Glu	24	Thr	27	Val	11
19	Ile	67	Met	19	Thr	15
20	Ser	51	Leu	33	Met	6
21	Ala	70	Pro	20	Ser	10
22	Ala	74	Gly	24	Pro	14
23	Val	42	Asp	11	Gly	14
24	Met	54	Gly	22	Asp	8
25	Lys	109	Val	20	Gly	15
26	Ile	54[b]	Gly	21	Asp	6
27	Phe	37	Pro	14	Gly	15
28	Asp	30	Glu	12	Pro	11
29	Ala	60	Leu	29	Glu	8
30	Ala	71	Met	11	Leu	16
31	Lys	30	Ala	18	Met	5
32	Ala	61	Ala	21	Leu	18
33	Pro	26	Val	17	Thr	9
34	Ile	42			Val	12
35	Gln	15				

[a] The yield of Ala in the first cycle was 94, 94, 16, and 5 pmol when the sequence was determined from subunits α_1, α_2, $\alpha_3 + \alpha_4$ (combined), and α_5, respectively. [b] From cycle 26 on, the data are from subunit β_2. [c] When the sequenced subunits were isolated from the same sample of complete enzyme, the yield of Ala in the first cycle was 61, 33, and 17 from subunit β_1, β_2, and β_3, respectively. [d] The yield of Phe in the first cycle was 21, 25, 19, and 9 pmol from subunit γ_1, γ_2, γ_3, and γ_4, respectively.

Gas-phase sequencing of subunit bands γ_1, γ_2, γ_3, and γ_4 revealed the identical sequence: F-S-Q-Q-T-I-P-P-S-A-K-Y-G-G-I-L-T-V-T-M-S-P-G-D-G-D-G-P-E-L-M-L-T-V. No differences in sequence were detected between the protein bands within each group. For many of the subsequent analyses, all of the bands within each subunit type have been pooled.

The sequencing data from α, β, and γ subunits agree with the previous finding that α and β chains each have alanine as the N-terminal amino acid, whereas phenylalanine is the N-terminal residue of the γ subunit. When the sequenced subunits were isolated from the same sample of complete enzyme, the total amounts of PTH-Ala measured in the first cycle for α_1–α_5 was 209 pmol, of PTH-Ala measured in the first cycle for β_1–β_3 was 111 pmol, and of PTH-Phe measured in the first cycle for γ_1–γ_4 was 74 pmol. This is reasonably consistent with the ratio of 2α:1β:1γ postulated previously (Ramachandran & Colman, 1980).

Although each subunit type has its own characteristic amino acid sequence, similar regions are observed in the α, β, and γ subunits. In the most favorable alignment, shown in Table II, it is apparent that the sequence containing residues 7–23 of the α subunit is most similar to the regions which include residues 17–33 of the β subunit and residues 18–34 of the γ subunit. Of these 17 positions, 10 amino acids are identical

Table II: Alignment of N-Terminal Sequences of α, β, and γ Subunits

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                          1     5      10          15          20          25          30          35
α                         A-G-G-V-K-T V-T-L-I-P-G-D-G-I-G-P-E-I-S-A-A-V M-K-I-F-D-A-A-K-A-P-I-Q

        1     5        10        15        20        25          30
β       A-S-R-S-Q-A-E-D-V-R-V-E-G-A-F-P V-T-M-L-P-G-D-G-V-G-P-E-L-M-A-A-V

       1     5        10        15       20          25          30
γ      F-S-Q-Q-T-I-P-P-S-A-K-Y-G-G-I-L-T V-T-M-S-P-G-D-G-D-G-P-E-L-M-L-T-V
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in all three subunit types. At most of the other positions, there are conservative substitutions (e.g., isoleucine for leucine, leucine for methionine). Over the entire region shown in Table II, 13 residues are identical between the α and β subunits; 13 residues are identical between the α and γ subunits; and 14 amino acids are identical between the β and γ subunits. Clearly, the three distinguishable subunits of the NAD-dependent isocitrate dehydrogenase are closely related.

Separation by HPLC of Tryptic Digests of Complete Enzyme and Separated Subunits. The multiple subunit types of the pig heart NAD-specific isocitrate dehydrogenase were originally proposed when it was recognized that the large number of tryptic peptides observed upon two-dimensional paper chromatography and high-voltage electrophoresis was greater than would be predicted from the 41 lysine plus arginine residues per average subunit of 40 000 daltons (Ramachandran & Colman, 1978). High-performance liquid chromatography affords a more effective preparative procedure for isolating these tryptic peptides. A complex peptide pattern was obtained when the complete enzyme, with its cysteine residues carboxymethylated, was digested with trypsin and then fractionated by HPLC on a reverse-phase C_{18} column equilibrated with 0.1% trifluoroacetic acid, using a gradient in acetonitrile (solvent system I).

The α, β, and γ subunits of NAD-dependent isocitrate dehydrogenase were separated by chromatofocusing in the presence of 6 M urea on a column of PBE-94 resin, as reported previously (Huang & Colman, 1989; Ehrlich & Colman, 1983). On this column, the subunits were eluted as peaks in their characteristic pH range: γ, 7.4–6.7; β, 6.3–6.0; and α, 5.4–5.1. These pH ranges for α, β, and γ subunits are close to those measured in the isoelectric focusing gel. The identities of the subunits collected from different pH ranges were further verified by isoelectric focusing in polyacrylamide gels. Small aliquots of the pooled fractions from each peak obtained by chromatofocusing were subjected to isoelectric focusing in polyacrylamide gels. The results indicated that the peaks obtained by chromatofocusing represent, respectively, the α, β, and γ subunits of the typical gel pattern.

A characteristic peptide pattern was obtained when each of the separated subunits was treated with iodoacetate, digested with trypsin, and fractionated by HPLC on the C_{18} column equilibrated with 0.1% trifluoroacetic acid. The tryptic maps obtained from subunits α, β, and γ are readily distinguished, and each is considerably less complex than the peptide pattern for the complete enzyme (data not shown, but provided to reviewers).

Separation by HPLC of ([^{14}C]Carboxymethyl)Cysteine-Containing Peptides from Complete Enzyme and Enzyme Subunits. The number of free sulfhydryl groups in pig heart NAD-dependent isocitrate dehydrogenase was determined by reaction with 5,5′-dithiobis(2-nitrobenzoate) as described under Experimental Procedures. The enzyme was found to have 6.0 free SH groups per average subunit of 40 000 daltons. It was

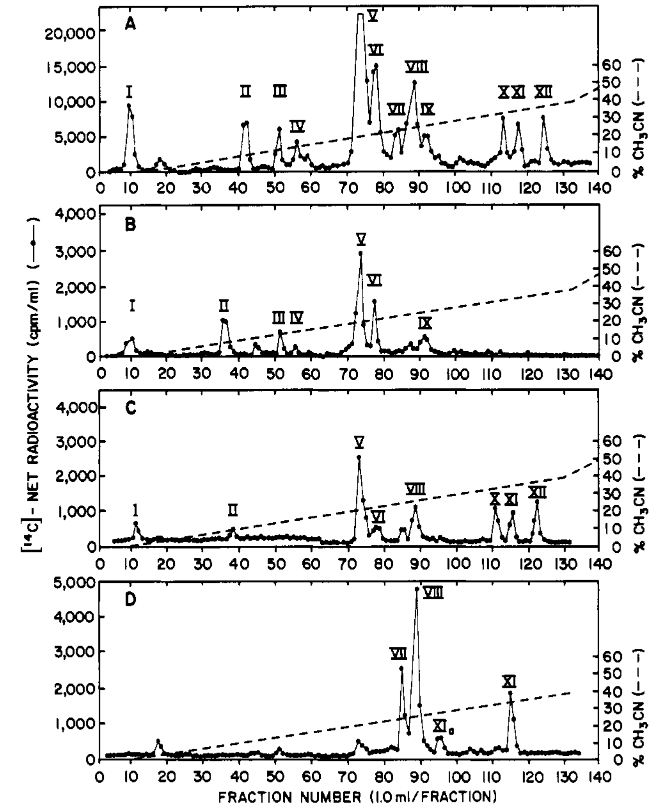

FIGURE 2: Distribution of radioactive peaks derived from tryptic digests of complete isocitrate dehydrogenase and its separated subunits which had been labeled by [^{14}C]iodoacetate. The tryptic digests were applied to a Vydac C_{18} column equilibrated with 0.1% trifluoroacetic acid and eluted at a flow rate of 1 mL/min using solvent system I, as described under Experimental Procedures. The samples used are as follows: (A) complete enzyme; (B) α subunit; (C) β subunit; and (D) γ subunit.

previously shown that all the enzyme subunits of NAD-dependent isocitrate dehydrogenase contain cysteine residues (Ramachandran & Colman, 1980). Therefore, it seemed that characterization of the cysteine peptides in the complete enzyme and separated subunits would provide another approach to ascertaining the similarity or distinctions among the subunits. Accordingly, either the complete enzyme or the individual subunits separated by chromatofocusing were carboxymethylated in 6 M urea using [^{14}C]iodoacetate prior to digestion by trypsin, as described under Experimental Procedures. The resultant peptides were fractionated by HPLC in 0.1% trifluoroacetate acid (solvent system I). The elution profile revealed 12 major ^{14}C-labeled peaks derived from the digest of the complete enzyme (Figure 2A). Each of these peaks was further purified by HPLC on the C_{18} column equilibrated with 20 mM ammonium acetate, pH 5.8 (solvent system II). Rechromatography of most of the peaks yielded a single, homogeneous radioactive peptide. The exceptions were peak V, which gave rise to three distinct labeled peptides

Table III: Amino Acid Sequences of Cysteine-Containing Peptides from Complete Enzyme

	amount of amino acid (pmol)						
cycle no.	II	III	IV	V_a	V_b	V_c	VI
1	Glu (1420)	CmCys (193)	Glu (403)	CmCys (1420)	Gly (1710)	Ala (4140)	Ile (3800)
2	Val (1590)	Arg (108)	Val (388)	Ser (1000)	Val (2020)	Asn (2370)	Glu (2240)
3	Ala (1950)	Glu (265)	Ala (466)	Asp (666)	Ile (1930)	Val (3360)	Thr (2950)
4	Glu (1260)	Val (387)	Glu (300)	Phe (1220)	Glu (1100)	Arg (1130)	Ala (3310)
5	Asn (1070)	Ala (491)	Asn (320)	Thr (1090)	CmCys (1270)	Pro (2520)	CmCys (2000)
6	CmCys (1010)	Glu (230)	CmCys (475)	Glu (617)	Leu (1270)	CmCys (2660)	Phe (2480)
7	Lys (944)	Asn (274)	Lys (473)	Glu (600)	Lys (661)	Val (2790)	Ala (2510)
8		CmCys (274)	Asp (210)	Ile (697)		Ser (1500)	Thr (1700)
9		Lys (274)	Ile (236)	CmCys (550)		Ile (1850)	Ile (1460)
10			Lys (255)	Arg (61)		Glu (1160)	Lys (1190)
11						Gly (1100)	
12						Tyr (1780)	
13						Lys (1280)	
14							
15							
16							
17							
18							
19							
20							
21							
22							
23							
24							
subunit source	α	α	α	α	β	α	α

	amount of amino acid (pmol)						
cycle no.	VII	$VIII_a$	$VIII_b$	IX	X	XI	XII
1	Ser (390)	Leu (1120)	Thr (598)	Phe (840)	Leu (170)	Asn (631)	Thr (498)
2	Ala (442)	Gly (514)	Ser (500)	Asn (177)	Gly (109)	Ile (736)	Ser (450)
3	CmCys (405)	Asp (269)	Leu (768)	Glu (612)	Asp (56)	Ala (1094)	Asp (251)
4	Val (317)	Gly (430)	Asp (227)	Met (1010)	Gly (51)	Asn (659)	Met (531)
5	Pro (358)	Leu (857)	Leu (732)	Tyr (611)	Leu (137)	Pro (646)	Gly (361)
6	Val (365)	Phe (525)	Tyr (493)	Leu (1110)	Phe (68)	Thr (531)	Gly (445)
7	Asp (194)	Leu (795)	Ala (529)	Asp (398)	Leu (95)	Ala (726)	Tyr (396)
8	Phe (384)	Gln (409)	Asn (363)	Thr (495)	Gln (55)	Thr (466)	Ala (548)
9	Glu (116)	CmCys (455)	Val (341)	Val (503)	CmCys (48)	Leu (836)	Thr (347)
10	Glu (305)	CmCys (455)	Ile (312)	CmCys (380)	CmCys (48)	Leu (936)	CmCys (322)
11	Val (201)	Lys (502)	His (87)	Leu (762)	Glu (40)	Ala (498)	Gln (296)
12	Val (61)		CmCys (300)	Asn (152)	Glu (44)	Ser (210)	Asp (186)
13	Val (227)		Lys (283)		Val (28)	CmCys (232)	Phe (258)
14	Ser (94)				Ala (33)	Met (254)	Thr (278)
15	Ser (33)				Glu (27)	Met (331)	Glu (119)
16	Asn (80)				Leu (60)	Leu (357)	Ala (242)
17	Ala (121)				Tyr (22)	Asp (110)	Val (188)
18	Asp (42)				Pro (22)	His (31)	Ile (143)
19	Glu (46)				Lys (7)	Leu (266)	Gly (143)
20	Glu (71)					Lys (62)	Ala (195)
21	Asp (41)						Leu (214)
22	Ile (16)						Ser (70)
23	Arg (18)						His (16)
24							Pro (46)
subunit source	γ	β, γ	γ	α	β	β, γ	β

(termed V_a, V_b, and V_c), and peak VIII, which yielded two discrete radioactive peptides (termed $VIII_a$ and $VIII_b$). A total of 15 ^{14}C-labeled peptides were thus identified from the proteolytic digest of complete enzyme. The amino acid sequence of each of these peptides was determined by gas-phase Edman degradation with representative results shown in Table III. Each peptide was shown to contain one or two ^{14}C-labeled (carboxymethyl)cysteines. Peak I was a mixture which appeared to contain radioactive CmCys-Lys and CmCys-Arg; these could have been derived from nontraditional digestion of peaks II, III, V_a, $VIII_a$, and $VIII_b$.

The subunits isolated by chromatofocusing were dialyzed separately to remove ampholytes, carboxymethylated with [^{14}C]iodoacetate, and digested with trypsin. To facilitate the identification of the subunit source of the specific ([^{14}C]-carboxymethyl)cysteine-containing peaks, the subunits were fractionated by HPLC in 0.1% TFA (system I) under the same conditions as those for the complete enzyme. Figure 2B–D compares the distribution of radioactive peaks derived from α, β, and γ subunits with those of the complete enzyme (Figure 2A). Seven major radioactive peaks were found in the chromatogram of the α subunit digest corresponding to peaks I–VI and IX of the complete enzyme. In the β subunit digest, five major radioactive peaks were detected, corresponding to peaks V, VIII, and X–XII. In the digest of the γ subunit, three major radioactive peaks were observed, corresponding to peaks VII, VIII, and XI of the complete enzyme. The peak designated as XI_a in Figure 2D was found to contain the sequence N-I-A-N-P-T-A-T-L-L-A-S-(Cm)C, which is the same as the first 13 residues of peptide XI (Table III). Peak XI_a is probably the minor product of an unusual cleavage of peptide XI by the trypsin preparation.

Alternatively, as described under Experimental Procedures, the complete enzyme was first carboxymethylated with

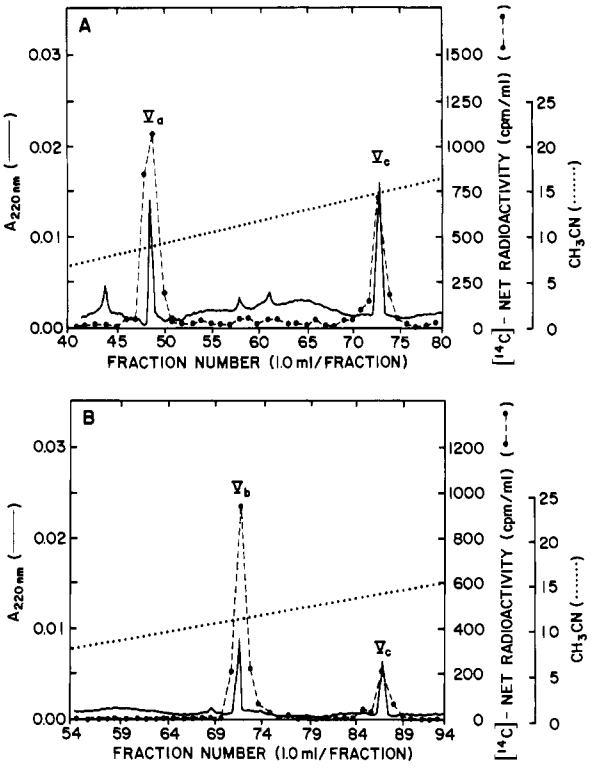

FIGURE 3: Further purification of peak V by HPLC in 20 mM ammonium acetate, pH 5.8. (A) Radioactive peak V from α subunit (Figure 2B, fractions 72 and 73) was subjected to HPLC on a Vydac C_{18} column equilibrated with 20 mM ammonium acetate, pH 5.8 (solvent C). The column was eluted at a flow rate of 1 mL/min for 10 min with solvent C, and a linear gradient was run to 60% solvent D (50% acetonitrile) in 130 min followed by a linear gradient to 100% solvent D in the next 30 min. (B) The corresponding radioactive peak V from β subunit (Figure 2C) was applied to the Vydac C_{18} column and eluted for 10 min with solvent C. A linear gradient was run to 45% solvent D in 130 min followed by a linear gradient to 100% solvent D in the next 30 min. The flow rate was 1 mL/min, and A_{220} (—), as well as radioactivity (---), was monitored. The linear gradient of acetonitrile is indicated (···). Peptide peaks V_a (fractions 48 and 49) and V_c (fraction 73) from α subunit and peptide peaks V_b (fraction 72) and V_c (fraction 87) from β subunit were pooled and lyophilized.

[^{14}C]iodoacetate, and the subunits were separated by isoelectric focusing in polyacrylamide gels containing a 6 M urea and transferred electrophoretically to Immobilon. The separated subunit types were eluted and incubated with trypsin, and the digests were fractionated by HPLC in 0.1% trifluoroacetic acid. The elution profiles at A_{220nm} and radioactive patterns of [^{14}C]CmCys-containing peaks for the three types of subunit were similar to those obtained for the subunits separated by chromatofocusing (Figure 2B–D).

Subunits α and β both exhibited peak V, but it is known that this peak includes three radioactive peptides when isolated from the digest of complete enzyme. Accordingly, peak V from the individual subunit digests was further fractionated by HPLC in ammonium acetate (solvent II). As illustrated in Figure 3A, the α subunit digest yields two [^{14}C]carboxymethyl-peptides: V_a at 8.6% acetonitrile and V_c at 15% acetonitrile. In contrast, peak V from the β subunit digest (shown in Figure 3B) contains only one predominant radioactive peak: V_b at 10.7% acetonitrile.

Subunits β and γ both exhibited peak VIII, but in the digest from the complete enzyme, this peak contains two peptides separable in ammonium acetate. Therefore, peak VIII from both the β and γ subunit digests were subjected to rechromatography in solvent system II. Figure 4 indicates that the γ subunit yields both peptides $VIII_a$ and $VIII_b$; the peak for $VIII_a$ contains more radioactivity primarily because it has two

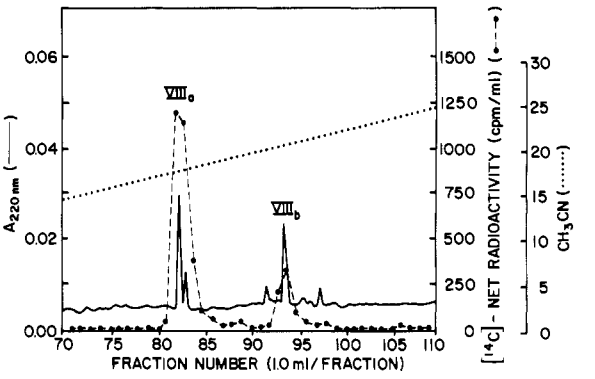

FIGURE 4: Further purification of peak VIII of γ subunit by HPLC in 20 mM ammonium acetate, pH 5.8. Radioactive peak VIII (Figure 2D) wa subjected to HPLC on a Vydac C_{18} column equilibrated with solvent C and eluted at 1.0 mL/min using solvent system II as described under Experimental Procedures. A_{220} (—) and radioactivity (---) were monitored, and the linear gradient in acetonitrile is indicated (···).

cysteines as compared to one for $VIII_b$. In contrast, the β subunit contains appreciable amounts of only peptide $VIII_a$.

Table III summarizes the subunit source of all the cysteine-containing peptides, as well as their sequences. The identification of peptides in the subunit digests was initially made by comparison with the mobility on HPLC of the peaks obtained from the digest of the complete enzyme. However, the identities of the subunit-derived peptides were then directly verified by sequencing.

The peptides of each subunit are compiled in Table IV to indicate the number of cysteines contained in each. In the case of the α subunit, the HPLC peaks I–IV and V_a (the longest of which is a decapeptide) represent a series of overlapping tryptic peptides; only II and V_a are the expected products of complete, specific digestion by trypsin. These have been assembled into the 20-residue peptide containing three cysteines that is shown in line 1 of Table IV. The α subunit has three additional unique cysteine-containing peptides. The total number of cysteines in the α subunit is therefore 6. Analysis of the β subunit reveals five cysteine-containing peptides, all of which are different from the four cysteine peptides of the α subunit. The second and third peptides of the β subunit (each of which includes two cysteines) have the first 10 residues in common; however, the third peptide has Glu instead of Lys at position 11 and therefore is not cleaved by trypsin at this position as is the second peptide. The five peptides of the β subunit listed in Table IV account for seven cysteines. The γ subunit exhibits only four cysteine-containing peptides, one of which includes two cysteines. Two of these peptides (the second and fourth) are found in the β as well as the γ subunit, but there are *no* cysteine-containing peptides common to α and γ subunits. The sequences listed for the γ subunit account for five distinguishable cysteines. Since the subunit composition of the pig heart NAD-dependent isocitrate dehydrogenase has been estimated as 2α:1β:1γ (Ramachandran & Colman, 1980), the cysteine content per average subunit was calculated as $[(2)(6) + 7 + 5]/4 = 6.0$. This value agrees with the experimental result of 6.0 free SH groups per average subunit as determined by reaction of isocitrate dehydrogenase with 5,5′-dithiobis(2-nitrobenzoate).

Discussion

Although previous work on the pig heart NAD-dependent isocitrate dehydrogenase had indicated that its subunits were not identical (Ramachandran & Colman, 1978, 1980), this paper presents direct evidence that the three subunit types exhibit distinct amino acid sequences. The N-terminal 33–

Table IV: Summary of Sequences of Cysteine-Containing Peptides and Their Subunit Locations

Number of Cysteines	HPLC Peaks	Sequences
		α-Subunit
3	I, II, III IV, V_a	[C]-S-D-F-T-E-E-I-[C]-R-E-V-A-E-N-[C]-K-D-I-K
1	V_c	A-N-V-R-P-[C]-V-S-I-E-G-Y-K
1	VI	I-E-T-A-[C]-F-A-T-I-K
1	IX	F-N-E-M-V-L-D-T-V-[C]-L-N
		β-Subunit
1	V_b	G-V-I-E-[C]-L-K
2	$VIII_a$, I	L-G-D-G-L-F-L-Q-[C-C]-K
2	X	L-G-D-G-L-F-L-Q-[C-C]-E-E-V-A-E-L-Y-P-K
1	XI	N-I-A-N-P-T-A-T-L-L-A-S-[C]-M-M-L-D-H-L-K
1	XII	T-S-D-M-G-G-Y-A-T-[C]-Q-D-F-T-E-A-V-I-G-A-L-S-H-P
		γ-Subunit
1	VII	S-A-[C]-V-P-V-D-F-E-E-V-V-V-S-S-N-A-D-E-E-D-I-R
2	$VIII_a$, I	L-G-D-G-L-F-L-Q-[C-C]-K
1	$VIII_b$, I	T-S-L-D-L-Y-A-N-V-I-H-[C]-K
1	XI	N-I-A-N-P-T-A-T-L-L-A-S-[C]-M-M-L-D-H-L-K

35-residue sequence of each of the α, β, and γ subunits is unique. However, if the amino-terminal sequence of the α subunit is displaced by 11 or 12 amino acids relative to that of the β and γ subunits (as in Table II), a 17 amino acid region of great similarity becomes apparent. Ten residues are invariant among the subunits, and with one exception, the others represent conservative substitutions. In most cases, the transformation from one amino acid to the other could be accomplished by a single base change in the codon (e.g., Leu to Met, Ile to Leu, Ile to Ser, Ile to Val, Val to Asp). The modern structure of isocitrate dehydrogenase may have arisen by gene duplication and separate evolution of the duplicate fragments. As determined by migration in polyacrylamide gels containing sodium dodecyl sulfate, the α subunit has a molecular weight of 39 000, while β and γ exhibit higher molecular weights of 41 000 (Ramachandran & Colman, 1978, 1980). In the best sequence alignment illustrated in Table II, the β and γ subunits are shown, respectively, with a 10 amino acid and 11 amino acid extension relative to the α subunit. These N-terminal extensions can account for 914 of additional molecular weight for the β subunit and 1056 for the γ subunit relative to the α subunit, an appreciable part of the total 2000 difference between α and the other subunits. In contrast to the relationship among the three subunits of pig heart NAD-specific isocitrate dehydrogenase, there is no apparent sequence similarity between any of these subunits and the first 30 residues at the amino-terminal end of the NADP-dependent isocitrate dehydrogenase of *Escherichia coli* (Thorsness & Koshland, 1987). Furthermore, there is no resemblance between the 30 N-terminal amino acids of pig heart NADP-specific isocitrate dehydrogenase (Smyth and Colman, unpublished data) and those of any of the subunits of the NAD-dependent enzyme from the same source.

Examination of the sequences of the cysteine-containing peptides provides additional evidence for the chemical diversity of the subunits of pig heart NAD-dependent isocitrate dehydrogenase. There are 6.0 free SH groups per average subunit of 40 000 daltons, but there are 15 distinguishable cysteines in peptides derived from the complete enzyme. On the basis of comparison of the sequences of the cysteine-containing peptides, β and γ are more similar to each other than either is to the α subunit. Two cysteine peptides are common to both β and γ subunits: that contained in HPLC peak $VIII_a$ (with two cysteines) and that of HPLC peak XI (with one cysteine). Furthermore, the peptide contained in peak $VIII_a$ (β and γ subunits) resembles closely that of peak X (β subunit) since the first 10 amino acids are identical. An earlier study of the affinity labeling of the allosteric ADP site by 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate led to the identification of L-G-D-G-L-F-L-Q as the modified peptide with the aspartate at position 3 as the target amino acid (Huang & Colman, 1989). It was recognized in that study that this modified peptide was part of a larger tryptic peptide (that of HPLC peak $VIII_a$) isolated from the γ subunit. It is now found that this tryptic peptide is present in the β subunit as well. In addition, the eight amino acid sequence L-G-D-G-L-F-L-Q is part of the peptide of peak X. Therefore, the modified peptide resulting from reaction with 2-[(4-bromo-2,3-dioxobutyl)thio]adenosine 5′-diphosphate was probably derived from both the β and γ subunits (Huang & Colman, 1989). These results imply that the β and γ subunits each has an allosteric binding site for ADP.

Comparison of the cysteinyl peptides of this pig heart NAD-dependent isocitrate dehydrogenase with those of the NADP-specific isocitrate dehydrogenase of the pig heart (Smyth et al., 1989) reveals no apparent similarities. On the other hand, there is some similarity between two of the six cysteine peptides of *E. coli* NADP-dependent isocitrate dehydrogenase (Thorsness & Koshland, 1987) and two of those of the pig heart NAD enzyme. A cysteine of the α subunit is comparable to the cysteine near the C-terminus of the *E. coli* enzyme:

E. coli	C^{405} - S - E - F - G - D
pig heart, peak V_a	C - S - D - F - T - E

The substitution of glutamate for aspartate may be considered conservative. A cysteine of the β and γ subunits resembles Cys^{301} of the *E. coli* enzyme:

E. coli	V - I - A - C^{301} - M - N - L
pig heart, peak XI	L - A - S - C - M - M - L

Perhaps a distant relationship between these two isocitrate dehydrogenases is indicated. However, there is no resemblance between the cysteine (γ subunit, peak VII) labeled by the substrate analogue 3-bromo-2-ketoglutarate in the active site of the pig heart NAD enzyme (Saha et al., 1989) and any of the cysteines of the *E. coli* enzyme, despite the fact that the bacterial enzyme is also inactivated by 3-bromo-2-ketoglutarate (Ehrlich and Colman, unpublished data). Other papers have reported inactivation of the NAD-dependent isocitrate dehydrogenase from pig heart by reaction of its cysteines with iodoacetate (Mauck & Colman, 1976) and from bovine heart by reaction of cysteines with 5,5′-dithiobis(2-nitrobenzoate), *N*-ethylmaleimide, and *p*-mercuribenzenesulfonate (Fan & Plaut, 1974). However, several cysteines were modified in each case, and they were not identified within peptides.

The sequences in the Swiss Protein Sequence Data Bank were scanned for similarity to the cysteinyl peptides of NAD-dependent isocitrate dehydrogenase using the Scansim program (PC Gene, Intelligenetics). This computer-based search revealed similarity between some of these peptides and sequences in other dehydrogenases. For example, there is some resemblance between the sequence of the peptide of peak XI from the β and γ subunits (L-A-S-C-M-M-L) and that of the essential Cys^{149} of glyceraldehyde-3-phosphate dehydrogenase (N-A-S-C^{149}-T-T-N), although the isocitrate dehydrogenase reaction would not be expected to involve a thioester intermediate such as that of the glyceraldehyde-3-phosphate dehydrogenase reaction (Harris & Waters, 1976). There also exists resemblance between certain of the cysteinyl peptides of horse liver alcohol dehydrogenase (Bränden et al., 1976; Eklund et al., 1984) and those of the NAD-dependent isocitrate dehydrogenase: between the peptide of peak $VIII_a$ from the β and γ subunits (Q-C-C-K) and Cys^{97} of alcohol dehydrogenase (Q-C^{97}-G-K); between the peptide of peak IX from the α subunit (L-D-T-V-C-L-I) and Cys^{170} of alcohol dehydrogenase (L-E-K-V-C^{170}-L-I); between the peptide of peak X from the β subunit (L-Q-C-C-E-E) and Cys^{281}, Cys^{282} of alcohol dehydrogenase (L-S-C^{281}-C-Q-E); and between the peptide of peak $VIII_b$ from the γ subunit (V-I-H-C-K) and Cys^{9} of alcohol dehydrogenase (V-I-K-C^{9}-K). In alcohol dehydrogenase, Cys^{97} is one of the ligands to the second zinc, Cys^{170} is close to the catalytic zinc binding site, and Cys^{281} is in the coenzyme binding domain. The NAD-dependent isocitrate dehydrogenase requires a divalent metal cation for catalysis and can use zinc, as well as manganese and magnesium, but the ligands to the metal ion are not known. Sequence comparisons have led many investigators to associate functions with particular regions of a subject protein. However, any postulated structure–function relationship must be evaluated experimentally by using biochemical and biophysical techniques.

The functional consequences of the structural diversity among the subunits of the pig heart NAD-dependent isocitrate dehydrogenase are not fully understood. It is well documented that the enzyme has one binding site per two subunits for isocitrate, Mn^{2+}, NAD, and NADH, as well as the allosteric activator ADP (Ehrlich & Colman, 1981, 1982). The enzyme can be dissociated to nearly inactive isolated subunits, followed by reconstitution to either $\alpha\beta$ or $\alpha\gamma$ dimers with substantial catalytic activity (Ehrlich & Colman, 1983). It seems likely that the β and γ subunits are functionally equivalent although structurally distinguishable, while the more divergent α subunits perform a different role in the intact enzyme. The catalytically active, allosteric tetrameric enzyme may then be envisioned as a dimer of dimers in which the $\alpha\beta$ and $\alpha\gamma$ dimers, though nonidentical, are functionally similar.

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