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Isolation and Sequence of a cDNA Encoding Porcine Mitochondrial NADP-Specific Isocitrate Dehydrogenase[†,‡]

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ABSTRACT: The cDNA for porcine mitochondrial NADP-specific isocitrate dehydrogenase was isolated from a λgt11 library using polymerase chain reaction. Translation of the DNA sequence gave a 413-residue amino acid sequence and a calculated molecular weight of 46 600 for the mature polypeptide. Previously determined peptide sequences for the amino terminus and for internal tryptic peptides were all contained within the translated sequence. The porcine protein was found to share 63% residue identity with yeast mitochondrial NADP-specific isocitrate dehydrogenase and to be immunoreactive with an antiserum against the yeast protein. Highly conserved regions include residues which have been implicated in substrate and cofactor binding in previous studies of the porcine enzyme. The two eucaryotic enzymes exhibit only minimal homology with the NADP-dependent isocitrate dehydrogenase from *Escherichia coli*, with the exception of a striking conservation of residues implicated in formation of the metal–isocitrate site of the procaryotic enzyme.

Isocitrate dehydrogenase (IDH)[1] catalyzes the oxidative decarboxylation of isocitrate to form α-ketoglutarate. While a single NADP-specific form of this enzyme is responsible for this reaction in *Escherichia coli* (Plaut, 1963; Ragland et al., 1966), multiple isozymes of IDH that vary in subunit structure and cofactor specificity have been reported for both lower and higher eucaryotes (Colman, 1968; Plaut, 1970; Keys & McAlister-Henn, 1990; Haselbeck & McAlister-Henn, 1991).

In the yeast *Saccharomyces cerevisiae*, distinct NAD- and NADP-specific isozymes are both located in the mitochondrial matrix (Keys & McAlister-Henn, 1990; Haselbeck & McAlister-Henn, 1991). The NAD-specific IDH has recently been shown to be a significant contributor to tricarboxylic acid (TCA) cycle activity (Keys & McAlister-Henn, 1990; Cupp & McAlister-Henn, 1991) and is allosterically responsive to the adenylate energy charge as well as to key TCA cycle metabolites (Hathaway & Atkinson, 1963; Barnes et al., 1971). The yeast mitochondrial NADP-specific isozyme has been proposed to provide an alternative TCA cycle activity (Machado et al., 1975), although its actual relative contribution to TCA cycle function or to other cellular processes remains unclear.

Partial characterization of the mitochondrial NADP-IDH (IDP1) of *S. cerevisiae* and cloning and sequencing of the corresponding structural gene have recently been described (Haselbeck & McAlister-Henn, 1991). IDP1 is a dimer of identical subunits with a subunit molecular weight of approximately 46 000. Its activity has been determined to not be allosterically regulated (Kornberg & Pricer, 1951). In these respects, yeast IDP1 resembles the IDH of *E. coli* (Reeves et al., 1972; Burke et al., 1974) as well as the mitochondrial NADP-IDH isolated from porcine heart tissue (Colman, 1968). While the primary structures of the *E. coli* and yeast enzymes are not significantly homologous (Thorsness & Koshland, 1987; Haselbeck & McAlister-Henn, 1991), key residues of *E. coli* IDH identified in crystallographic studies to be involved in the binding of isocitrate may also be conserved in IDP1.

Comparison of amino acid sequences obtained from tryptic peptides of porcine mitochondrial NADP-IDH (Smyth & Colman, 1991) with the predicted amino acid sequence of

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[1] Abbreviations: IDH, isocitrate dehydrogenase; TCA, tricarboxylic acid cycle; PCR, polymerase chain reaction; NEM, *N*-ethylmaleimide.

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IDP1 (Haselbeck & McAlister-Henn, 1991) suggests that the two eucaryotic enzymes may be structurally very similar. The enzymatic function if the porcine enzyme has been extensively characterized by physical and biochemical techniques [reviewed in Colman (1989)], but full determination of catalytic or cofactor binding structures has been hampered by lack of a complete primary sequence. We describe here the cloning and sequencing of a cDNA encoding the entire mature porcine NADP-IDH polypeptide and comparison of the amino acid sequence with that previously determined for the analogous enzyme from yeast.

Experimental Procedures

Strains. E. coli strain Y1090 (Young & Davis, 1983) was used for amplification and hybridization of λgt11 cDNA libraries and strain DH5αF′ (Raleigh et al., 1988) for plasmid amplifications.

Recombinant DNA Methods. Oligonucleotides used as primers for polymerase chain reaction or nucleotide sequence analysis were obtained from Operon Technologies, Inc. (Santa Monica, CA). PCR was conducted using a commercial kit (Perkin-Elmer/Cetus) with 1 μg of DNA prepared from a λgt11 cDNA library from porcine heart tissue (Clontech) and 10 nmol of oligonucleotide primers. An Erincomp Temperature Cycler was used to repeat the following cycle 40 times: 94 °C for 1 min, 37 °C for 1 min, and 72 °C for 3 min.

DNA fragments were purified from low-melt agarose gels for subcloning or for preparation of ^{32}P-labeled probes by the random primer method (Feinberg & Vogelstein, 1983). Subcloning, hybridization screens of λgt11 libraries, and preparation of DNA from purified λgt11 plaques were conducted using methods described by Maniatis et al. (1982).

For DNA sequence analysis, DNA fragments were subcloned in both orientations into plasmid pBS(−) (Strategene). Single-stranded DNA sequencing was conducted using the method described by Sanger et al. (1977) with a Sequenase II sequencing kit obtained from U.S. Biochemical Corp.

Western Blot Analysis. For immunoblot analysis, yeast and porcine NADP-IDHs purified as previously described (Haselbeck & McAlister-Henn, 1991; Smyth & Colman, 1991) were electrophoresed on 10% polyacrylamide/sodium dodecyl sulfate gels and electroblotted to Immobilon poly(vinylidene difluoride) filters (Millipore). The filters were incubated with a 1:100 dilution of rabbit anti-yeast NADP-IDH antiserum and ^{125}I-labeled protein A as previously described (Haselbeck & McAlister-Henn, 1991).

Results

Cloning of the cDNA Encoding Porcine NADP-IDH by Polymerase Chain Reaction. The sequences for several cysteine-containing tryptic peptides of porcine NADP-IDH and a 30-residue sequence from the amino terminus of the intact polypeptide have previously been reported (Smyth & Colman, 1991). The amino-terminal sequence and the sequences of certain of the tryptic fragments were found to be homologous with discrete portions of the predicted amino acid sequence of yeast mitochondrial NADP-IDH (IDP1), and alignment of these peptides with the IDP1 protein sequence allowed putative mapping of their relative locations within the porcine protein.

Of these tryptic fragments, two were chosen for synthesis of oppositely oriented oligonucleotides based on the least possible degeneracy obtainable for use as polymerase chain reaction (PCR) primers. The underlined portions of the peptide sequences CATITPEAR and AHSCFQYAIQK (cf. Figure 2) yielded the degenerate oligonucleotides 5′-AT-(TCA)AC(TCAG)CC(TCAG)GA(TC)GA(AG)GC-3′ and the oppositely oriented 5′-TT(TC)TG(AGT)AT(ACTG)CC-(AG)TA(TC)TG(AG)AA-3′, respectively. The oligonucleotides were used with a λgt11 cDNA library from porcine heart for amplification by PCR as described in Experimental Procedures. Above a background of nonspecific amplification, a unique 0.4-kbp amplification product was obtained from DNA purified from the porcine cDNA library and not from a control reaction containing λgt11 vector DNA. The 0.4-kbp amplification product was purified and reamplification of the resulting DNA yielded only the 0.4-kbp product. After further gel purification, the DNA fragment was subcloned into the *Sma*I restriction site of plasmid pBS(−) for nucleotide sequence analysis.

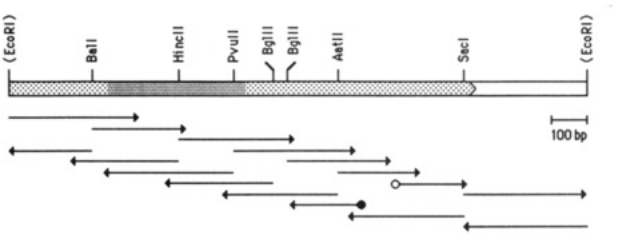

FIGURE 1: Partial restriction map and sequencing strategy of a cDNA encoding porcine mitochondrial NADP-IDH. Flanking *Eco*RI restriction sites indicated by parentheses are due to linkers used in construction of the λgt11 library. Arrows indicate the starting points and extent of nucleotide sequence obtained from individual reactions. Sequences denoted by arrows emanating from circles were performed using oligonucleotide primers based on previously determined sequences. The open reading frame determined by translation of the entire DNA sequence of the 1.6-kbp cDNA subclone is indicated by the stippled region of the restriction map. The area of darker stippling represents the location of the 0.4-kbp PCR amplification product.

Translation of the DNA sequence obtained for the 0.4-kbp insert gave an open reading frame with 65% residue identity with the corresponding portion of the yeast IDP1 protein sequence. The translated 0.4-kbp sequence also contained the previously unmapped porcine tryptic peptide sequence EPIICK (Smyth & Colman, 1991). This was compelling evidence that the 0.4-kbp amplification product actually represented a partial subclone of a cDNA encoding porcine mitochondrial NADP-IDH.

The 0.4-kbp DNA fragment was used to prepare a ^{32}P-labeled probe for hybridization screening of the porcine λgt11 cDNA library as described in Experimental Procedures. Screening of approximately 3×10^6 plaques produced two isolates that hybridized with the probe. DNA samples prepared from the two isolates were digested with *Eco*RI, resulting in excision of single fragments of 0.6 and 1.6 kbp, respectively. The larger *Eco*RI fragment was used for further characterization. After being subcloned in both orientations into the polylinker *Eco*RI site of plasmid pBS(−), the 1.6-kbp porcine cDNA was subjected to partial restriction mapping, with the results shown in Figure 1.

Nucleotide Sequence Analysis. The strategy employed to sequence the 1.6-kbp porcine cDNA is illustrated in Figure 1. A single open reading frame was found which abuts the 5′ *Eco*RI restriction site and extends through nucleotide number 1266. The entire DNA sequence and the amino acid sequence of the translated open reading frame are shown in Figure 2.

The amino-terminal residues of the mature porcine NADP-IDH as previously determined by protein sequence analysis are found within the 5′ end of the translated open reading frame and are preceded by eight residues that may represent a portion of the mitochondrial targeting sequence. Since the initiator methionine is not evident, the full prese-

```
 TT GCA AGA GCA GCC GCG CGC CAC TAT GCC GAC CAG AGG ATC AAG GTG GCG AAG   53
    A  R  A  A  A  A  R  H  Y  A  D  Q  R  I  K  V  A  K                 9

CCG GTG GTG GAG ATG GAC GGT GAT GAG ATG ACC CGA ATC ATC TGG CAG TTC ATC  107
P  V  V  E  M  D  G  D  E  M  T  R  I  I  W  Q  F  I                   27

AAG GAG AAG CTC ATC CTG CCC CAC GTG GAT GTC CAG CTC AAG TAT TTT GAC CTG  161
K  E  K  L  I  L  P  H  V  D  V  Q  L  K  Y  F  D  L                   45

GGG CTC CCA AAC CGG GAC CAG ACC AAC GAT CAG GTC ACC ATC GAC TCC GCC TTG  215
G  L  P  N  R  D  Q  T  N  D  Q  V  T  I  D  S  A  L                   63

GCC ACC CAG AAG TAC AGC GTG GCT GTC AAG TGT GCC ACC ATC ACC CCC GAT GAG  269
A  T  Q  K  Y  S  V  A  V  K  C  A  T  I  T  P  D  E                   81

GCC CGT GTG GAA GAG TTC AAG CTG AAG AAG ATG TGG AAG AGT CCC AAT GGA ACC  323
A  R  V  E  E  F  K  L  K  K  M  W  K  S  P  N  G  T                   99

ATC CGG AAC ATC CTC GGG GGG ACT GTT TTC CGG GAG CCC ATC ATC TGC AAG AAC  377
I  R  N  I  L  G  G  T  V  F  R  E  P  I  I  C  K  N                  117

ATC CCA CGC CTT GTG CCT GGC TGG ACC AAG CCC ATC ACC ATC GGC AGG CAC GCT  431
I  P  R  L  V  P  G  W  T  K  P  I  T  I  G  R  H  A                  135

CAC GGC GAC CAG TAC AAG GCC ACA GAC TTT GTG GTT GAC CGG GCG GGC ACG TTC  485
H  G  D  Q  Y  K  A  T  D  F  V  V  D  R  A  G  T  F                  153

AAG ATT GTC TTC ACC CCA AAG GAC GGC AGC AGT GCC AAG CAG TGG GAA GTG TAC  539
K  I  V  F  T  P  K  D  G  S  S  A  K  Q  W  E  V  Y                  171

AAC TTC CCC GCA GGC GGC GTG GGC ATG GGC ATG TAC AAC ACA GAT GAG TCC ATC  593
N  F  P  A  G  G  V  G  M  G  M  Y  N  T  D  E  S  I                  189

TCA GGG TTC GCG CAC AGC TGC TTC CAG TAC GCC ATC CAG AAG AAG TGG CCG CTC  647
S  G  F  A  H  S  C  F  Q  Y  A  I  Q  K  K  W  P  L                  207

TAC ATG AGC ACC AAG AAT ACC ATT CTG AAA GCC TAC GAC GGG CGC TTC AAA GAC  701
Y  M  S  T  K  N  T  I  L  K  A  Y  D  G  R  F  K  D                  225

ATC TTC CAG GAG ATC TTT GAG AAG CAC TAT AAG ACC GAT TTC GAC AAG TAT AAG  755
I  F  Q  E  I  F  E  K  H  Y  K  T  D  F  D  K  Y  K                  243

ATC TGG TAT GAG CAC CGG CTC ATT GAT GAC ATG GTG GCT CAG GTC CTC AAG TCT  809
I  W  Y  E  H  R  L  I  D  D  M  V  A  Q  V  L  K  S                  261

TCG GGC GGC TTC GTG TGG GCC TGC AAG AAC TAC GAT GGA GAC GTG CAG TCG GAC  863
S  G  G  F  V  W  A  C  K  N  Y  D  G  D  V  Q  S  D                  279

ATC CTG GCC CAG GGC TTT GGC TCC CTT GGC CTG ATG ACG TCT GTG CTG GTC TGC  917
I  L  A  Q  G  F  G  S  L  G  L  M  T  S  V  L  V  C                  297

CCG GAT GGC AAG ACC ATC GAA GCT GAG GCT GCT CAT GGG ACA GTC ACC CGC CAC  971
P  D  G  K  T  I  E  A  E  A  A  H  G  T  V  T  R  H                  315

TAT AGG GAG CAC CAG AAG GGC CGG CCT ACC AGC ACC AAC CCC ATC GCC AGC ATC 1025
Y  R  E  H  Q  K  G  R  P  T  S  T  N  P  I  A  S  I                  333

TTT GCC TGG ACG CGT GGC CTG GAG CAC CGG GGG AAG TTG GAC GGA AAC CAA GAC 1079
F  A  W  T  R  G  L  E  H  R  G  K  L  D  G  N  Q  D                  351

CTG ATC AGG TTT GCT CAG ACT CTG GAG AAG GTG TGT GTT GAG ACA GTA GAG AGC 1133
L  I  R  F  A  Q  T  L  E  K  V  C  V  E  T  V  E  S                  369

GGA GCC ATG ACC AAG GAC CTG GCG GGC TGT ATC CAT GGC CTC AGC AAT GTG AAG 1187
G  A  M  T  K  D  L  A  G  C  I  H  G  L  S  N  V  K                  387

CTG AAC GAG CAC TTC CTG AAC ACC TCG GAC TTC CTG GAC ACC ATC AAA AGC AAC 1241
L  N  E  H  F  L  N  T  S  D  F  L  D  T  I  K  S  N                  405

CTG GAC AGA GCT CTG GGC CGG CAG TAG                                    1268
L  D  R  A  L  G  R  Q  End                                            413

CGGGGTGGGAGCGCTGTGGGCTGCCCTGGTGGCCGGGCCGGAGCCCAGCGGGTGGCCGCAGTTGCCCACCTC 1340
CCTCCAGCACCACTTGGCGAGGGTGAGCCTCTCAGCCCCTCTTAGGAGGCCTCTCCAGGGGCTATTTTTTTA 1412
TAAGCAAGATGTTTTTAAAGTATCTGTGTGTTTCCCCTCATGGTGACATGAGGCAGGAACAGAGTGTTTTAC 1484
CTCAGCCAGTGCGTGGTTCTGCGTACTGTAATTTATATTGTCCTCGGAACACATGGTGCCATGTTTAGCTAC 1556
TAAAAAGCTCTTCACAAAAAAAAAAAAA                                           1585
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FIGURE 2: DNA sequence and translated amino acid sequence of the 1.6-kbp cDNA isolate. The alanine at the amino terminus of the mature protein is indicated with a box and is designated residue 1. Previously determined amino acid sequences for the amino terminus, internal tryptic peptides (Smyth & Colman, 1991), and other peptides are indicated by underlining. Peptide sequences including residues 77–82 and 196–203 were used to plan synthesis of oligonucleotides for PCR as described in the text.

quence is presumably truncated in this cDNA isolate. The mature protein sequence predicted from the open reading frame represents a 413-residue polypeptide with a molecular weight of 46 600, which is similar in size to the 412-residue, 46 300 molecular weight yeast IDP1 translation product (Haselbeck & McAlister-Henn, 1991).

Following the stop codon of the translated open reading frame is a 347-bp sequence that ends in 14 consecutive adenine nucleotides. While this poly(A) sequence may represent polyadenylation of the porcine NADP-IDH transcript, proximal sequences 5′ to this region seem to lack a canonical polyadenylation site (Wickens, 1990). It is therefore possible that this stretch of adenine nucleotides is encoded by the original transcript and that the actual polyadenylation site and poly(A) tail are not represented in this isolate.

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PIG:     ADQRIKVAKPV--VEMDGDEMTRIIWQFIKEKLILPHVDVQ----------LKYF    43
YEAST:   FS-KIKVKQPV--VELDGDEMTRIIWDKIKKKLILPYLDVD----------LKYY    42
E.COLI:  MESKVVVPAQGKKITLQNGKLN------VPENPIIPYIEGDGIGVDVTPAMLKVV    49

         DLGLPNRDQTNDQVT----IDSALATQKYSVAVKCATITPDEARVEEFKLKKMWK    94
         DLSVESRDATSDKIT----QDAAEAIKKYGVGIKCATITPDEARVKEFNLHKMWK    93
         DAAVEKAYKGERKISWMEIYTGEKSTQVYGQDVWLPAETLDLIREYRVAIKGPLT   104

         SP-NGTIRNILGGTVFREPI---ICKNIPRLVPGWTKPI--------TIGRHAHG   137
         SP-NGTIRNILGGTVFREPI---VIPRIPRLVPRWEKPI--------IIGRHAHG   136
         TPVGGGIRSL--NVALRQELDLYICLRPVRYYQGTPSPVKHPELTDMVIFRENSE   157

         DQYKATDFVVDRAGTFKIVFTPKDGSSAKQ--WEVYNFPAG-GVGMGMYNTDESI   189
         DQYKATDTLIPGPGSLELVYKPSDPTTAQPQTLKVYDYKGS-GVAMAMYNTDESI   190
         DIYAGIEWKADSADAEKVIKFLREEMGVKK-----IRFPEHCGIGIKPCS-EEGT   206

         SGFAHSCFQYAIQKKWP-LYMSTKNTILKAYDGRFKDIFQEIFEKHYKTDFDKYK   243
         EGFAHSSFKLAIDKKLN-LFLSTKNTILKKYDGRFKDIFQEVYEAQYKSKFEQLG   244
         KRLVRAAIEYAIANDRDSVTLVHKGNIMKFTEGAFKDWGYQLAREEFGGELIDGG   261

         IWYEHR--------LIDDMVA-----QVLKSSGGF-VWACKNYDGDVQSDILAQG   284
         IHYEHR--------LIDDMVA-----QMIKSKGGF-IMALKNYDGDVQSDIVAQG   285
         PWLKVKNPNTGKEIVIKDVIADAFLQQILLRPAEYDVIACMNLNGDYISDALAAQ   316

         FGSLGLMTSVLVCPDGKTIEAEAAHGTVTRHYREHQKGRPTSTNPIASIFAWTRG   339
         FGSLGLMTSILVTPDGKTFESEAAHGTVTRHYRKYQKGEETSTNSIASIFAWSRG   340
         VGGIGIAPGANIGDECALFEA--THGTAPK-YAGQDKVNPGSIILSAEMMLHRMG   368

         LEHRGKLDGNQDLIRFAQTLEKVCVETVE-SGAMTKDLA-GCIHGLSNVKLNEHF   392
         LLKRGELDNTPALCKFANILESATLNTVQQDGIMTKDLALACGNNERSA-----Y   390
         WTEAADL----------------IVKGMEGAINAKTVTYDFERLMDGAKL----   402

         LNTSDFLDTIKSNLDRAL-GRQ   413
         VTTEEFLDAVEKRLQKEIKSIE   412
         LKCSEFGDAIIENM--------   416
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FIGURE 3: Alignment of amino acid sequences of porcine, yeast, and *E. coli* NADP-IDHs. The primary sequences of the mature polypeptides of porcine and yeast mitochondrial NADH-IDHs (Haselbeck & McAlister-Henn, 1991) and of *E. coli* IDH (Thorsness & Koshland, 1987) were aligned to maximize relatedness. Identical residues are indicated by colons. The alignment was performed using the CLUSTAL program of PC/GENE by the method of Higgins and Sharp (1988).

As indicated by underlining in Figure 2, all previously reported peptide sequences (Smyth & Colman, 1991) and additional sequences which have been determined by peptide sequencing are in agreement with the translated cDNA sequence. Seven cysteine residues are included in the complete sequence shown in Figure 2, whereas only six cysteine-containing peptide sequences have previously been reported (Smyth & Colman, 1991). The additional Cys 297 would be expected in a 31-residue tryptic peptide which is likely to have been lost during the HPLC procedures used for peptide purification. The content of seven cysteines is consistent with the total number of cysteines in the protein determined by analytical methodology (Smyth & Colman, 1991).

Comparison of the Primary Structures of Yeast and Porcine Mitochondrial and of E. coli NADP-IDHs. The polypeptide sequences of mature porcine and yeast mitochondrial NADP-IDHs and of *E. coli* IDH were aligned to maximize relatedness as shown in Figure 3. The two eucaryotic enzymes display 63% residue identity overall; much of this identity is found in long contiguous stretches within the first three-fourths of the molecules, while the carboxy-terminal fourths of the

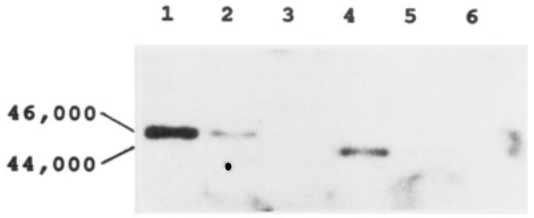

FIGURE 4: Western blot analysis of porcine and yeast NADP-IDHs. Equimolar amounts (0.5 μg in lanes 1 and 4, 0.1 μg in lanes 2 and 5, and 0.02 μg in lanes 3 and 6) of purified yeast (lanes 1–3) and porcine (lanes 4–6) NADP-IDHs were electrophoresed for immunoblot analysis using anti-yeast NADP-IDH antiserum as described in Experimental Procedures. Immunoreactivity of the porcine enzyme was estimated by densitometry to be approximately 5-fold lower than that of the yeast enzyme. No immunoreactivity was evident for either protein with the use of preimmune serum at the same dilution as anti-yeast NADP-IDH antiserum. Although the calculated molecular weights of the two polypeptides are approximately equivalent (46 000), the porcine peptide migrates faster than predicted in this analysis, exhibiting an apparent molecular weight of approximately 44 000.

molecules are comparatively dissimilar. The general extent of similarity is also reflected in the ability of a rabbit polyclonal antiserum raised against yeast IDP1 to cross-react with porcine NADP-IDH as determined by Western blot analysis (Figure 4), while it fails to recognize *E. coli* IDH (data not shown).

In previous studies, several of the cysteine residues in specific tryptic peptides were reported to be protected from modification by the reagent *N*-ethylmaleimide (NEM) when the native enzyme was treated in the presence of isocitrate plus Mn^{2+} (Smyth & Colman, 1991). In particular, protection of the cysteine residue in the porcine peptide SSGGFVWACK (residues 261–270) is believed to prevent inactivation by NEM, suggesting an important role for this region in isocitrate/Mn^{2+} binding. One of the cysteine-containing peptides (DLAGCIHGLSNVK, residues 375–387) that was not protected from NEM modification in the same study is located near the carboxy terminus. Residues in this region have previously been implicated in formation of the NADP binding pocket by affinity labeling of the peptide with a reactive NADP analogue (Bailey & Colman, 1987). These peptides are represented by homologous regions in the yeast sequence, suggesting that conserved residues may be involved in substrate and cofactor binding in both enzymes.

When the amino acid sequences of porcine and yeast NADP-IDHs are aligned with that reported for *E. coli* IDH (Thorsness & Koshland, 1987; Figure 3), identities in approximately 14% of the residue positions are obtained. Despite this relatively low degree of homology, residues of the procaryotic enzyme previously implicated by crystallographic analysis to interact with bound isocitrate plus Mg^{2+} (Hurley et al., 1991) are highly conserved in the eucaryotic enzymes. *E. coli* residues Arg 119, Arg 153, Tyr 160, Lys 230, Asp 307, Asp 311, and Glu 336 correspond to porcine residues Arg 110, Arg 133, Tyr 140, Lys 212, Asp 275, Asp 279, and Glu 304 or 306, respectively, and with identical residues at similar positions in the yeast enzyme. Asp 275 in the porcine enzyme corresponds to a metal-binding residue in the bacterial enzyme and is located near a porcine peptide containing residues implicated in a similar function. Interestingly, despite similarities in cofactor specificity, very few of the residues in the bacterial enzyme which have been implicated in NADP binding (Hurley et al., 1991) can be aligned with conserved residues in the eucaryotic proteins. Three residues in this category, Gly 320, His 339, and Tyr 345, of the *E. coli* enzyme are identical in this alignment with residues Gly 289, His 309, and Tyr 316 in the porcine enzyme. The NADP-binding domain described for the bacterial enzyme is unique among nicotinamide nucleotide-binding proteins (Hurley et al., 1991). Therefore, future comparisons of the binding sites in the eucaryotic enzymes may help define critical features of this motif.

Discussion

This work describes cloning and sequence analysis of a porcine cDNA encoding mitochondrial NADP-IDH. The predicted primary structure, total amino acid content, and subunit molecular weight are all highly similar to those of the analogous yeast enzyme; moreover, regions of the porcine enzyme that have been implicated in substrate and cofactor binding are highly conserved in the yeast enzyme. These results, along with strong similarities in native structure, cofactor specificity, and cellular compartmentation, suggest that the metabolic roles of porcine and yeast NADP-IDH may also be similar and that yeast is a good model to gain a greater understanding of this role in both higher and lower eucaryotes using the powerful genetic and biochemical manipulations available with this system.

Another goal in determining the amino acid sequence of the porcine enzyme is to facilitate determination of three-dimensional structure. This has been accomplished for the NADP-IDH of *E. coli* (Hurley et al., 1989, 1991), and key residues involved in substrate binding as determined by this method appear to be conserved in the procaryotic and eucaryotic enzymes. This suggests that other functional and structural similarities may become evident upon determination of the three-dimensional structure of either or both of the eucaryotic enzymes despite their overall lack of similarity to *E. coli* IDH at the amino acid sequence level.

Acknowledgments

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Binding Specificities of Actinomycin D to Non-Self-Complementary -XGCY- Tetranucleotide Sequences†

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ABSTRACT: Studies on the binding specificity of actinomycin D (ACTD) to tetranucleotide sequences of the form -XGCY- have been extended to include the non-self-complementary sequences. ACTD binding characteristics are investigated by equilibrium, kinetic, and thermal denaturation for decameric duplexes d(ATA-XGCY-ATA)-d(TAT-Y′GCX′-TAT), where X and Y are complementary to X′ and Y′, respectively, but not to each other. The results indicate that when X = G or Y = C, the oligomers exhibit significantly weaker ACTD binding affinities, smaller melting temperature increases upon drug binding, and faster SDS-induced ACTD dissociation rates than the other sequences. Estimated binding constants at 18.5 °C for decameric duplexes containing -AGCA-/-TGCT-, -AGCG-/-CGCT-, or -CGCA-/-TGCG- are in the range of 4–9 μM^{-1}, whereas for the ones containing -GGCT-/-AGCC-, -GGCA-/-TGCC-, or -GGCG-/-CGCC- they range from 0.6 to 2 μM^{-1}. In contrast to the characteristic SDS-induced ACTD dissociation times of 600–1000 s for the stronger binding sites, the sequences containing X = G or Y = C exhibit at least an order of magnitude faster dissociation kinetics. These observations are further supported by the induced CD results and fluorescence measurements with 7-amino-ACTD. The findings from these non-self-complementary -XGCY- tetranucleotide sequences are consistent with those found earlier for the self-complementary counterparts, and they together clearly demonstrate that a base sequence alteration adjacent to the GC site can have a profound effect on the ACTD binding as well as dissociation characteristics, likely a consequence of subtle conformational alterations near the binding site. Our results on the SDS-driven dissociation kinetics support the site-heterogeneity model of drug dissociation from native DNA and further extend the heterogeneity concept beyond the dinucleotide sequence level.

Actinomycin D (ACTD) is an antitumor antibiotic that contains a 2-aminophenoxazin-3-one chromophore and two cyclic pentapeptide lactones. The biological activity of ACTD is believed to be the consequence of its ability to bind to duplex DNA which results in the inhibition of DNA-dependent RNA polymerase activities. Earlier binding studies with synthetic polynucleotides (Goldberg et al., 1962; Wells & Larson, 1970) had established the guanine specificity of this drug. Detailed spectroscopic and hydrodynamic studies led Muller and Crothers (1968) and Waring (1970) to conclude that ACTD binds to DNA via insertion of its phenoxazone chromophore between the DNA base pairs. On the basis of their X-ray diffraction results of a 2:1 complex of deoxyguanosine with ACTD, Sobell and Jain (1972) subsequently proposed a binding model of intercalation at the dG-dC sequence with specific hydrogen bonding between the 2-amino group of guanine and the carbonyl oxygen of threonine in the peptide rings. NMR studies (Patel, 1974; Krugh et al., 1977; Brown et al., 1984) using oligonucleotides containing a dG-dC sequence had generally agreed with such a binding model. DNase I footprinting experiments (Lane et al., 1983; Scamrov & Beabealashvilli, 1983; Fox & Waring, 1984a) have further confirmed the dG-dC binding specificity of ACTD. Comparative studies with oligonucleotides of specific lengths and sequences (Wilson et al., 1986; Chen, 1988a) have conclusively demonstrated that the binding of ACTD to the dC-dG sequence is much weaker than that to dG-dC. Thus, at the dinucleotide level, the binding preference of ACTD at the dG-dC sequence appears to be firmly established.

Aside from being supportive of the fact that dG-dC is the strong binding site, Aivasashvilli and Beabealashvilli (1983)

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