Cloning of a cDNA encoding bovine mitochondrial NAD⁺-specific isocitrate dehydrogenase and structural comparison with its isoenzymes from different species

Tae-Lin HUH,* Jae-Ha RYU,† Jae-Wook HUH,‡ Ha-Chin SUNG,† Il-Ung OH,* Byoung J. SONG‡ and Richard L. VEECH‡§

*Department of Genetic Engineering, College of Natural Sciences, Kyungpook National University, Taegu, Korea,

†Department of Genetic Engineering, College of Agricultural Sciences, Korea University, Seoul, Korea,

and ‡Laboratory of Metabolism and Molecular Biology, National Institute on Alcohol Abuse and Alcoholism, 12501 Washington Avenue, Rockville, MD 20852, U.S.A.

Mitochondrial NAD⁺-specific isocitrate dehydrogenase (IDP) was co-purified with the pyruvate dehydrogenase complex from bovine kidney mitochondria. The determination of its N-terminal 16-amino-acid sequence revealed that it is highly similar to the IDP from yeast. A cDNA clone (1.8 kb long) encoding this protein was isolated from a bovine kidney λ gt11 cDNA library using a synthetic oligodeoxynucleotide. The deduced protein sequence of this cDNA clone rendered a precursor protein of 452 amino-acid residues (50830 Da) and a mature protein of 413 amino-acid residues (46519 Da). It is 100% identical to the internal tryptic peptide sequences of the autologous form from pig heart and 62% similar to that from yeast. However, it shares

little similarity with the mitochondrial NAD⁺-specific isoenzyme from yeast. Structural analyses of the deduced proteins of IDP isoenzymes from different species indicated that similarity exists in certain regions, which may represent the common domains for the active sites or coenzyme-binding sites. In Northern-blot analysis, one species of mRNA (about 2.2 kb for both bovine and human) was hybridized with a ³²P-labelled cDNA probe. Southern-blot analysis of genomic DNAs verified simple patterns of hybridization with this cDNA. These results strongly indicate that the mitochondrial IDP may be derived from a single gene family which does not appear to be closely related to that of the NAD⁺-specific isoenzyme.

INTRODUCTION

The isocitrate dehydrogenases (ICDHs; EC 1.1.1.41 and EC 1.1.1.42) catalyse oxidative decarboxylation of isocitrate to α -oxoglutarate and require either NAD⁺ or NADP⁺, producing NADH or NADPH respectively. It is well known that there are three species of ICDH isoenzymes, depending on the cofactor requirement and subcellular localization [1]. Because of its role in intermediary metabolism and energy production [2], ICDH has been extensively studied with respect to its isoenzymes, regulation, kinetics, and its presence in other species including prokaryotes [3]. In *Escherichia coli*, isocitrate is converted into α -oxoglutarate by only one type of enzyme, NADP⁺-specific ICDH (IDP), the catalytic activity of which is covalently regulated by phosphorylation and dephosphorylation [2,4,5]. The phosphorylation of the enzyme decreases its catalytic activity by inhibiting the substrate binding [6], bypassing the tricarboxylic acid cycle to proportionate carbons needed for growth and energy production [7]. In contrast, the catalytic activity of NAD⁺-specific ICDH is known to be modulated by allosteric regulators in mammalian systems [8–10]. In yeast mitochondria, NAD⁺-specific ICDH appears to play a major role in the tricarboxylic acid cycle, while the role of the NADP⁺-specific enzyme, either cytosolic or mitochondrial, remains unestablished [11]. In mammalian systems, a substantial amount of IDP activity was also reported to be associated with the mitochondria, especially in heart and kidney [12]. Despite numerous biochemical and kinetic characterizations of this enzyme, its structure and relationship to

the other isoenzymes of ICDH, especially in mammalian systems, have not been addressed. We here report the complete nucleotide and deduced protein sequences of bovine mitochondrial IDP. We also analysed the structural relatedness among six different isoenzymes of ICDH and one isopropyl malate dehydrogenase (IPMDH) from other species.

MATERIALS AND METHODS

Reagents

Restriction enzymes, T₄ DNA ligase, and molecular-mass standards (for protein, DNA and RNA) were purchased from Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). SDS, acrylamide, and bisacrylamide were purchased from Bio-Rad Biochemicals (Richmond, CA, U.S.A.). The nick translation kit, 5'-end labelling kit, polynucleotide kinase, and terminal transferase were from Boehringer-Mannheim Biochemicals (Indianapolis, IN, U.S.A.). [α -³²P]dCTP (specific radioactivity 3000 Ci/mmol), [γ -³²P]ATP (specific radioactivity 3000 Ci/mmol), deoxyadenosine-5'- α -thiotriphosphate dATP[α -³⁵S] (specific radioactivity 500 Ci/mmol), and Gene Screen membrane were obtained from DuPont-New England Nuclear (Boston, MA, U.S.A.). Polyvinylidene difluoride (Immobilon) membranes were obtained from Millipore Inc. (Bedford, MA, U.S.A.). Polyethylene glycol (8000 Da) and *p*-hydroxymercuriphenyl-sulphonate were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were of the highest grade commercially available.

Abbreviations used: ICDH, isocitrate dehydrogenase; IDP, NADP⁺-specific ICDH; Y-IDP1, yeast mitochondrial IDP; Y-IDH2, yeast NAD⁺-specific ICDH [23]; IPMDH, NAD⁺-specific isopropyl malate dehydrogenase [24].

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§ To whom correspondence should be addressed.

Figure 1 SDS/PAGE for partially purified IDP

IDP was co-purified with the pyruvate dehydrogenase (PDH) kinase from bovine kidney mitochondria as described in the text. Each protein was further separated by SDS/PAGE and stained with Coomassie Blue. Several subunits of the PDH multienzyme complex and ICDH are indicated. The protein standards used (but not shown) were BSA (68 kDa) and ovalbumin (45 kDa).

Partial purification of NAD(P)-specific ICDH and determination of its N-terminal amino-acid sequence

Fresh bovine kidney was purchased from a local slaughterhouse and the cortex fraction was collected for the preparation of mitochondria by the method of Pettit and Reed [13]. The mitochondrial IDP of bovine kidney was co-purified with the pyruvate dehydrogenase kinase by the method of Stepp and Reed [14]. Partially purified mitochondrial IDP, along with the pyruvate dehydrogenase kinase, was subjected to SDS/PAGE and electrically transferred to polyvinylidene difluoride (Immobilon) membranes. The membranes were briefly stained with 0.1% [in 1% (v/v) acetic acid solution] Ponceau S Red dye (Eastman Kodak Company, Rochester, NY, U.S.A.) before cutting out the protein bands. The membrane strip containing the protein band was then dried and sent to the Microchemistry Laboratory, Harvard University for N-terminal amino-acid-sequence analysis. Another strip was used to confirm the data of amino-acid-sequence analysis in our laboratory, using a protein sequencer Model 473 with Sequence Software program (Model 610A) (Applied Biosystems Inc., Foster City, CA, U.S.A.).

Screening of a cDNA library and nucleotide sequencing

Based on the N-terminal 16-amino-acid sequence of bovine mitochondrial IDP, a 48-mer oligodeoxynucleotide was synthesized by a DNA synthesizer, PCR-Mate, Model 300 (Applied Biosystems Inc.) and purified before use. The anti-sense

Figure 2 Nucleotide and deduced protein sequences of the bovine mitochondrial IDP cDNA insert

The entire nucleotide and deduced protein sequences of the bovine mitochondrial IDP cDNA insert are shown. The canonical initiation codon, ATG and the termination codon, TAG were marked in boxes. The amino-acid residues determined by N-terminal amino-acid-sequence analysis are underlined.

sequence of the synthesized oligomer was 5'-GCCATCCATCT-CCACCACAGGCTTGGCCACCTTGATCCGCTTGTCAGC-3' following the best-codon usage [15]. The oligodeoxynucleotide was radiolabelled at the 5' position with [γ - 32 P]ATP and T₄ polynucleotide kinase using an end-labelling kit. A λ gt11 cDNA library from bovine kidney (Clontech, Palo Alto, CA, U.S.A.) was screened with a 32 P-labelled oligomer probe by plaque hybridization. The conditions of hybridization and washing were the same as described by Davis et al. [16]. Positive plaques from hybridization were further purified until independent plaques were obtained. The 5'-portion of the largest cDNA insert (1.8 kb) was gradually deleted using a kit (Erase-a-Base from Promega Corp., Madison, WI, U.S.A.) according to the protocol suggested by the manufacturer. The DNAs of different sizes were then subcloned into the pGEM plasmid (Promega) and used for

Figure 3 Homology comparison among isoenzymes of IDPs from different species

The deduced amino-acid sequences of five IDP isoenzymes from bovine kidney mitochondria (B-IDPm), pig mitochondria (P-IDPm) [20], yeast (Y-IDP1) [17], *E. coli* (E-ICDH) [21], and *T. thermophilus* (T-ICDH) [22] were aligned using the Microgenie software. The sequences of two other structurally related proteins, Y-IDH2 [23] and *T. thermophilus* IPMDH [24] were also included. The amino-acid residues identical to those of B-IDPm are represented in stippled boxes. Amino-acid residues in darker shades indicate the identical amino acids among the five isoenzymes of ICDH mentioned above and the NAD⁺-specific isoenzyme from yeast (Y-IDH2) [23]. The amino-acid sequences of six tryptic peptides of IDP from pig heart mitochondria [19] are indicated as P(I), P(II), P(III), P(IV), P(V), and P(VI). Three regions of highly conserved amino-acid residues between B-IDPm and other proteins are also indicated by I, II, and III. Spaces inserted for the alignment are indicated by hyphens.

(a)

B-IDPm	A D K R I K V A K P F V V E M D G D E M T - - - R I I W Q F I K E K L I L P . - - - H V D	37
P-IDPm	A D Q R I K V A K P F V V E M D G D E M T - - - R I I W Q F I K E K L I L P . - - - H V D	37
Y-IDP1	A F S K I K V K Q P F V V E L D G D E M T - - - R I I W D K I K E K L I L P . - - - Y L D	37
E-ICDH	- - M E S K V - - - V V P A Q G G K K I T L Q N G K L N V P E N P I I P Y I E G D G I G V D	40
T-ICDH	- - - - - P L I T T E T G K K M H V L E D G R K L I T V I P G D G I G P E	32
Y-IDH2	- - - - A T V K Q - - - P S I G R Y T G K - - P N P S T G K Y T V S F I E G D G I G P E	35
T-IPMDH	E P T R K G V E E A E A V L R A L D E A E - G L G L A Y E V F P F - G G A A I D A F G E - P F F	14

B-IDPm	V - - - Q L X Y F D - - - L G L P N R D Q T N D Q V T I - D S A L A T Q K Y S V A V K C A	75
P-IDPm	V - - - Q L X Y F D - - - L G L P N R D Q T N D Q V T I - D S A L A T Q K Y S V A V K C A	75
Y-IDP1	V - - - D L X Y F D - - - L S V E S R D A T S D K I T Q - D A A E A I K K Y G V G I K C A	75
E-ICDH	V T P A M L X Y V D A A A V E K A Y K G E R K I S W M E I Y T G E K S T Q V R Y G Q D V W L P	85
T-ICDH	C V E A T L X Y V L E A A - K A P - - L A Y E V R E - A G A S - - V F R R G I A S G V P	69
Y-IDH2	I S K S A L X I F S A A N V P I E W E S C D V S P I F V N G L T T - - - - - I P	70
T-IPMDH	T E A A L X V L R A L D E A E - G L G L A Y E V F P F - G G A A I D A F G E - P F F	54

B-IDPm	T I T P D E A R V E E F K L K - - - K M W K S P - N G T I - - R N I L G G T V F R E - -	111
P-IDPm	T I T P D E A R V E E F K L K - - - K M W K S P - N G T I - - R N I L G G T V F R E - -	111
Y-IDP1	T I T P D E A R V K E F N L H - - - K M W K S P - N G T I - - R N I L G G T V F R E - -	111
E-ICDH	A E T I D L I R E Y R V A I K - - - G P L T T P V G G G I - - R - S L N V A L R Q E L D	123
T-ICDH	Q E T I E S I R K T R V V L K - - - G P L E T P V G Y G E - - K - S A N V T L R K L F E	107
Y-IDH2	D P A V Q S I T K N L V A L K - - - G P L A T P I G K G H - - R - S L N L T L R K T F G	108
T-IPMDH	E P T R K G V E E A E A V L L G S V G G P K W D G L P R K I S P E - T G L L S L R K S Q D	98

B-IDPm	P I I C K N I P R - - - L - - V P G W T K - - - P - I T - - I G R H A H G D Q Y K A T D	144
P-IDPm	P I I C K N I P R - - - L - - V P G W T K - - - P - I T - - I G R H A H G D Q Y K A T D	144
Y-IDP1	P I I V I P R I P R - - - L - - V P R W E K - - - P - I I - - I G R H A H G D Q Y K A T D	144
E-ICDH	L Y I C L R P V R Y Y Q G - - T P S P W K H - - P E L T D M V I F R E N S E D I Y A G I E	164
T-ICDH	T Y A N V R P V R E F P N - - V P T P Y A G - - - R G I D L V L I R E N V E D L Y A G I E	147
Y-IDH2	L F A N V R P A K S I E G - - F K T T Y E N - - - V D L V L I R E N T E G E Y S G I E	146
T-IPMDH	L F A N L R P A K V F P G L E R L S P L K E E I A R G V D V L I V R E L T G G I Y F G E P	143

B-IDPm	F V V D - R A G T F - K V V - - F T P K D - - G S G P K E W E Y V N F P A G G V G M G - M	182
P-IDPm	F V V D - R A G T F - K I V - - F T P K D - - G S S A K Q W E Y V N F P A G G V G M G - M	182
Y-IDP1	T L I P - G P G S L - E L V - - Y K P S D P T T A Q P Q T L K V Y D F Y K G S G V A M A - M	184
E-ICDH	W K A D - S A D A E - K V I - - K F L R E - - E M G V K - - K I R E F - E H C G I G I K	199
T-ICDH	H M Q T P S V A Q T L K L I S W K G S E - - - - - - - - - H I V C - P G V V Q S I K	157
Y-IDH2	R G M - - - S E A - E A W N T E	168
T-IPMDH	R G M - - - S E A - E A W N T E	155

B-IDPm	Y N T D E S I S G F A H S C F Q Y A I - Q K K W P L Y M S T K N T I L K A Y D G R F K D -	225
P-IDPm	Y M T D E S I S G F A H S C F Q Y A I - Q K K W P L Y M S T K N T I L K A Y D G R F K D -	225
Y-IDP1	Y N T D E S I E G F A H S S F K L A I - D K K L N V L F L S T K N T I L K K Y D G R F K D -	227
E-ICDH	P C S E E G T K R L R V A A I E A I A N D R D S V T L V H K G N I M K F T E G A F K D W	244
T-ICDH	- - - - - K I V R F A F E L A R A E G R K K V H C A T K S N I Q M L A E G P - K R A	203
Y-IDH2	L I T R D A S E R V I R Y A F E Y A R A I G R P R V I V H K S T I L Q R L A D G L F V N V	203
T-IPMDH	R Y S K P E V E R V A R V A F E A A R K - R R K H V V S V D K A N V L E V G E - F W R K T	198

B-IDPm	I F Q - A - - I F - - - - E K H Y K T E F D K H - K - - I W Y E H R L I D D M V A - -	256
P-IDPm	I F Q - E - - I F - - - - E K H Y K T D F D K Y - K - - I W Y E H R L I D D M V A - -	256
Y-IDP1	I F Q - E - - V Y - - - - E A Q Y K S K F E Q L - G - - I H Y E H R L I D D M V A - -	258
E-ICDH	G Y Q L A R E E F G G - - - E L I D G G P W L K V - K N P N T G K E I V I K D V I A D A	284
T-ICDH	F E Q V A Q - E Y P D I E A V H I I V D N	223
Y-IDH2	A K E L S K A Y P E V S L E T E L I D N S V - L K V V T N P - - - - - G Y P D V A L E H Q Y V D A	232
T-IPMDH	V E E V G R - G Y P D V A L E H Q Y V D A	218

(b)

B-IDPm	- - Q - - V L K S S G G F V W A C K N Y D G D V Q S D I L A Q - G F G S L G L M T S V L V	296
P-IDPm	- - Q - - V L K S S G G F V W A C K N Y D G D V Q S D I L A Q - G F G S L G L M T S V L V	296
Y-IDP1	- - Q - - M I K S G G G F I M A L K N Y D G D V Q S D I V A Q - Q V G G I G I - A P G A N	298
E-ICDH	F L Q Q I L L R P A E Y D V I A C M N L N G D V Y I S D A L A A - Q V G G I G I - A P G A N	327
T-ICDH	A A H Q L V K R P E Q F E I V T T N M N G D I L S D L T S G - L I G G I G F - A P S A N	266
Y-IDH2	- - - - - S A Y T D A V S V C P N L Y G D I L S D L N S G L S A G S L G L - T P S A N	269
T-IPMDH	M A M H L V R S P A R F D V V V T G N I F I G D I L S D L A S V - L P G S L G L - L P S A S	261

B-IDPm	C P D G K T I E A E A A H G T V T R H Y R E H Q K G R P T S T N P I A S I F A W T R G L E	341
P-IDPm	C P D G K T I E A E A A H G T V T R H Y R E H Q K G R P T S T N P I A S I F A W T R G L E	341
Y-IDP1	T P D G K T F E S E A A H G T V T R H Y R K Y Q K G E E T S T N S I A S I F A W S	343
E-ICDH	I G D - E C A L F E A T H G T - A P K Y A G Q D K N V P G S - - I I L S - - A - E M M L R	365
T-ICDH	I G N - E V A I F E A V H G S - A P K Y A G K N V I N P T A - - V L L S - - A - V M M L R	304
Y-IDH2	I G H - K I S I F E A V H G S - A P D I A G Q D K A N P T A - - L L L S - - S - V M M L R	307
T-IPMDH	L G R - G T P V F E P V H G S - A P D I A G K G I A N P T A - - A I L S - - A - A M M L R	299

B-IDPm	H R - G K L D G N Q D L I R F A Q T L E K V C V E T V - E S G A M T K D L A G - C - I H -	381
P-IDPm	H R - G K L D G N Q D L I R F A Q T L E K V C V E T V - E S G A M T K D L A G - C - I H -	381
Y-IDP1	K R - G E L D N T P A L C K F A N I L E S A T L N T V Q Q D G I M T K D L A L A C G N N -	386
E-ICDH	H M - G - W T E A A D L I - V K G M E G A I N A K T V - - - - T Y D F E R - L - M D -	398
T-ICDH	Y L - E - E F A T A D L I - E N A L Y T L E E G R V - - - - - L T G D V V - G - Y D R	338
Y-IDH2	H M - G - L T N H A D Q I - Q N A V L S T I A S G P E - - - - - N R - - - - T - G D -	336
T-IPMDH	H A F G - L V E L A R K V - E D A V A K A L L E T P P - - - - - P - - - - - - D L -	327

B-IDPm	G L S N V K L N E H F L - N T S D F L - D T I K S N L D R A L G Q Q	413
P-IDPm	G L S N V K L N E H F L - N T S D F L - D T I K S N L D R A L G R Q	413
Y-IDP1	E R S A V V T T E E F L D A V E K R L Q K E I K S I E	413
E-ICDH	G - - - A K L - - - - L - K C S E F G - D A I I E N M	416
T-ICDH	G - - - A K T - - - - - - - T E Y T - E A I I Q N L G K T P R K T	360
Y-IDH2	L - - - A G T - - - - A - T T S S F T - E A V I K R L	354
T-IPMDH	G - - - G S A - - - - G - T E A F T A - T V L R H L A	354

nucleotide sequencing for both strands. Nucleotide sequencing was performed by the dideoxynucleotide chain termination method using a dideoxyDNA sequencing kit with Sequenase (United States Biochemicals, Cleveland, OH, U.S.A.). The nucleotide sequence data of mitochondrial IDP were assembled and analysed with the computer software, Microgenie (Beckman Instruments, Menlo Park, CA, U.S.A.).

Northern mRNA blot analyses

Poly(A⁺) RNA (2 µg/well) from both bovine and human heart (from Clontech) were separated on a 0.66 M formaldehyde/1 % agarose gel by the method of Davis et al. [16], transferred to Gene Screen membrane (New England Nuclear), and baked for 2 h at 80 °C in a vacuum oven. Prehybridization and hybridization were processed at 60 °C overnight in a 6 × SSC solution (1 × SSC contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) with 5 × Denhardt's solution (1 × Denhardt's solution contains 0.02 % each of Ficoll, polyvinylpyrrolidone, and BSA), 100 µg/ml salmon sperm DNA and 0.1 % SDS. After hybridization with the ³²P-labelled cDNA probe (1.8 kb) for bovine mitochondrial IDP, the membranes were washed four to five times with 1 × SSC containing 0.1 % SDS at 60 °C. When necessary, a second series of washings with 0.2 × SSC containing 0.1 % SDS was performed. The optimum conditions for washing were empirically determined by comparing the positive signals with background labelling. Washed membranes were exposed to X-ray films (Eastman Kodak Company, Rochester, NY, U.S.A.) for autoradiography at -80 °C with the aid of intensifying screens (DuPont Chemicals Company, Wilmington, DE, U.S.A.).

Southern-blot analysis

High-molecular-mass human genomic DNAs were purchased from Clontech and digested overnight at 37 °C with five restriction enzymes and separated on a 0.8 % agarose gel before blotting on to Gene Screen membrane. The membrane was baked in a vacuum oven for 2 h at 80 °C and then hybridized with ³²P-labelled mitochondrial IDP cDNA probe (1.8 kb) overnight at 60 °C. The hybridization and washing conditions were almost identical to those described for Northern mRNA blot analysis.

RESULTS

Partial purification of mitochondrial IDP and N-terminal amino-acid-sequence determination

During the purification of the pyruvate dehydrogenase complex and its kinase from bovine kidney mitochondria, IDP was co-purified with the pyruvate dehydrogenase kinase (Figure 1). The purification procedure for the latter enzyme [14] included isolation and solubilization of mitochondria, two cycles of polyethylene glycol precipitation, ultracentrifugation, gel filtration on Sephacryl HR S-400, and *p*-hydroxymercuriphenylsulphonate precipitation to enrich further pyruvate dehydrogenase kinase. However, the partially purified pyruvate dehydrogenase kinase (Figure 1) contained another contaminating protein band migrating as 45 kDa on SDS/PAGE. The N-terminal 16-amino-acid sequence of the unknown protein band was: NH₂-Ala-Asp-Lys-Arg-Ile-Lys-Val-Ala-Lys-Pro-Val-Val-Glu-Met-Asp-Gly-COOH. When this peptide sequence was compared with the protein databank, it matched that of the mitochondrial IDP from yeast, IDP1 [17].

Isolation and characterization of cDNA clones for bovine mitochondrial IDP

To isolate a cDNA clone for mitochondrial IDP, a λgt11 cDNA library from bovine kidney was screened with a ³²P-labelled 48-mer oligodeoxynucleotide by plaque hybridization. After screening more than one million plaque-forming units of recombinant DNA clones, ten positive independent clones with cDNA inserts were identified. When each positive plaque was further purified, the size of the cDNA inserts in these clones ranged from 0.6 kb to 1.8 kb long. Eight out of ten clones were cross-hybridized with two different ³²P-labelled cDNA inserts, indicating the structural relatedness among these cDNA clones. The largest cDNA insert (size about 1.8 kb) was subcloned to plasmid pGEM7 and designated pIDPm.

Nucleotide and deduced protein sequences of bovine mitochondrial IDP

The entire nucleotide sequence of the largest clone, pIDPm, was determined for both strands as described in the Materials and methods section. The sequencing data (Figure 2) revealed that our cDNA clone has neither a potential polyadenylation signal (AATAAA) nor a poly(A⁺) tail on its 3'-untranslated region. It is thus unlikely that it represents a full-length cDNA clone. However, it contained an open reading frame for the entire protein coding region of IDP consisting of 1359 bp, starting with the initiation codon, ATG, and ending with the termination codon, TAG. As summarized in Figure 2, the deduced protein of mitochondrial IDP exactly matched the 16 N-terminal amino-acid sequences determined by the protein sequencing and contained a mature protein of 413 amino acids (46519 Da). The precursor protein of 452 amino acids (50830 Da) contained an additional 39 amino acids, including six basic and 19 hydrophobic amino acids as a putative mitochondrial leader sequence similar to that reported earlier [18]. The calculated value for the molecular mass of the mature protein of mitochondrial IDP thus agrees well with the estimated monomeric size of 45 kDa on SDS/PAGE (Figure 1).

Structural analysis of bovine mitochondrial IDP

The deduced amino-acid sequence of bovine mitochondrial IDP (Figure 3) was 100 % identical to those of six Cys-containing tryptic peptides of pig heart IDP [19] [marked as P(I)-P(VI) in Figure 3]. The bovine mitochondrial IDP showed 97 %, 62 %, 25 %, and 24 % amino-acid-sequence similarity to the IDPs from pig mitochondria [20], yeast mitochondria (IDP1) [17], *E. coli* [21], and *Thermus thermophilus* [22], respectively. The similarities between IDP isoenzymes from various species exist in certain distinct regions, which may be related to the common domains for active sites or regulatory sites for the enzyme. In contrast, it is less similar (25 %) to the deduced protein sequence of the mitochondrial NAD⁺-specific isoenzyme from yeast, Y-IDH2 [23], as compared with 62 % similarity to yeast mitochondrial IDP1 [17]. In addition, it is also 17 % similar to NAD⁺-specific IPMDH from *T. thermophilus* [24].

Characterization of mitochondrial IDP mRNA

In order to determine how many species of mitochondrial IDP mRNA are present in mammalian tissues, a Northern mRNA blot was performed on RNA from both bovine and human heart. As shown in Figure 4, one species of mRNA (about 2.2 kb in size) was hybridized with the ³²P-labelled cDNA probe for

Figure 4 Northern mRNA blot analysis for bovine mitochondrial IDP

Poly(A⁺) RNA samples (2 µg/lane) from bovine and human heart were subjected to denaturing agarose gel electrophoresis, transferred to Gene Screen membranes and hybridized with the ³²P-labelled cDNA probe for bovine mitochondrial IDP as described in the Materials and methods section. The arrow represents the hybridized mRNA for pIDPm. Sizes of RNA ladders are also designated on the left.

bovine mitochondrial IDP. Only one species of mRNA band (2.2 kb) was also recognized by the same probe in other human tissues such as brain, liver, and lung (data not shown), indicating one major species of mRNA present for the mitochondrial IDP gene in both bovine and human tissues.

Genomic DNA Southern-blot analysis for the bovine mitochondrial IDP gene

To verify whether bovine mitochondrial IDP has closely related family members including mitochondrial NAD⁺-specific ICDH and cytosolic IDP, Southern-blot analysis of genomic DNA was performed. Genomic DNA Southern-blot analysis (Figure 5) revealed that only a few fragments were hybridized with the cDNA probe for bovine mitochondrial IDP. From the sum of the sizes of DNA fragments generated by *Eco*RI cleavage (23 kb and 9.5 kb), the total length for the mitochondrial IDP gene would be approx. 32.5 kb, at most. The relatively simple pattern of hybridization indicates that the mitochondrial IDP gene may be derived from a single gene family, which may not be closely related to that for the NAD⁺-specific isoenzyme.

DISCUSSION

Mammalian systems contain at least three distinct ICDH isoenzymes, the subcellular distribution of which is dependent

Figure 5 Southern-blot analysis for the bovine mitochondrial IDP gene

Human genomic DNA (10 µg/lane) was digested with restriction enzymes as indicated: *Bgl*II, *Pst*I, *Bam*HI, *Hind*III, and *Eco*RI. The DNA fragments were separated on 0.8% agarose gel, transferred to Gene Screen membrane and then subjected to genomic Southern-blot analysis as detailed in the Materials and methods section. Sizes of DNA markers are indicated on the left.

upon the tissues and species examined [1,12]. Although they have been purified to homogeneity from various sources [10,25–27], information regarding their structural differences was not known. In this report, we demonstrated the co-purification of mitochondrial IDP with pyruvate dehydrogenase kinase, where the latter enzyme was purified more than 300-fold from the mitochondrial extract. The results thus suggest that bovine mitochondrial IDP may tightly associate or interact with the pyruvate dehydrogenase complex. Although we do not know the physiological significance of the co-purification (presumably tight association) of these mitochondrial enzymes, similar cases of association of other mitochondrial enzymes have been reported previously [28,29].

We also described the isolation of a cDNA clone for mitochondrial IDP from bovine kidney and the determination of its complete nucleotide and deduced protein sequences. The high concordance between the amino-acid sequences of bovine mitochondrial IDP (current study) and the tryptic peptides [19] and deduced protein from a cDNA [20] of pig heart mitochondrial IDP showed that our clone represents the mitochondrial IDP. The results of mRNA Northern-blot and genomic DNA Southern-blot analyses and the relatively low similarity to yeast NAD⁺-specific isoenzyme IDH2 [23] strongly indicated that the bovine mitochondrial IDP gene may be derived from a single gene which appears structurally less related to that of NAD⁺-specific ICDH. Although we do not know the relationship between the mitochondrial IDP and its cytosolic counterpart, it is likely that these two NAD⁺-specific isoenzymes are derived from two different genes, based on the lack of cross-immunoreactivity [26], different tissue-specific expression [12,30] and different subcellular localization [1,12]. A similar multilocus system for IDP isoenzymes has been reported in fish, *Fundulus heteroclitus* [31].

The structural analysis of the deduced protein sequence of bovine mitochondrial IDP and the comparison with other ICDH isoenzymes from different species and the structurally related *T. thermophilus* IPMDH protein [24] raise several questions. First,

two of these proteins, *E. coli* ICDH and *T. thermophilus* IPMDH [21,24,32], do not appear to relate structurally to other well-known NAD⁺-dependent dehydrogenases, originally reported by Rossmann et al. [33]. We do not know whether bovine mitochondrial IDP is topologically distinct from 'Rossmann-fold'-type dehydrogenases. However, this notion has to be verified by further structural analysis using techniques such as X-ray crystallography. Secondly, bovine mitochondrial IDP does not have the serine residue as a phosphorylation site equivalent to Ser¹¹³ of IDP in *E. coli* [21]. This fact could explain why mammalian IDP may not be regulated by covalent modification. Thirdly, the structural similarity between bovine mitochondrial IDP and the other four IDP isoenzymes exists only in certain regions (regions I, II, and III shown in Figure 3). These conserved regions of structural similarity may represent the catalytic pockets as proposed from the three-dimensional structure of the *E. coli* ICDH enzyme [32,34]. Surprisingly, these three regions of similar structure found in the five IDPs are also conserved in NAD⁺-specific ICDH (yeast IDH2) and *T. thermophilus* IPMDH [24] (darker shades in Figure 3). These highly conserved regions with Lys²¹² (region I) and His³⁰⁹ of bovine mitochondrial IDP (region III) thus appear to constitute active sites for both NADP⁺- and NAD⁺-dependent ICDH or IPMDH. Fourthly, Hurley et al. [32,34] have suggested that certain charged and hydrophobic amino-acid residues may be responsible for the binding of isocitrate and metal ions. These amino-acid residues needed for the binding of substrate and bivalent cations appear to be conserved among the five IDP isoenzymes examined: in bovine mitochondrial IDP there are seven positively charged amino acids (Lys⁴¹, Arg⁸³, Arg¹²⁰, Arg¹³³, Lys²¹², Lys²¹⁷ and Lys²²⁴), six hydrophobic amino acids (Leu⁴⁰, Tyr¹⁴⁰, Ile²¹⁵, Tyr³¹⁶, Leu³⁴⁰ and Leu³⁵²), four negatively charged amino acids (Asp¹³⁸, Asp²⁷⁶, Asp²⁷⁹ and Glu³⁰⁶), and one His³⁰⁹. Fifthly, recent studies [35,36] suggested that structurally conserved glycine residues (Gly-Xaa-Gly-Xaa-Xaa-Gly-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Xaa-Gly) may be responsible for the interaction of the pyrophosphate moiety of NAD⁺ or NADP⁺. These conserved glycine residues are found in bovine mitochondrial IDP, pig mitochondrial IDP, and yeast mitochondrial IDP1 (Gly²⁸⁴, Gly²⁸⁶, Gly²⁸⁹, and Gly³⁰⁰ in Figure 3). However, it is difficult to conclude whether these glycine residues are really involved in the binding of the pyrophosphate moiety because of the absence of the first and fourth glycine in the following: *E. coli* ICDH, *T. thermophilus* ICDH, yeast IDH2 and *T. thermophilus* IPMDH. Finally, none of IDP isoenzymes examined contained the conserved amino-acid sequence of Arg-Xaa-Xaa-Xaa-Xaa-Arg, known as a motif for the interaction with the 2'-phosphate group of NADP⁺, which is found in other NADP⁺-dependent dehydrogenases [36]. However, two lysine residues (Lys²¹⁷ and Lys²²⁴) of bovine mitochondrial IDP are conserved in all IDP isoenzymes (pig mitochondrial IDP, yeast IDP1, *E. coli* ICDH and *T. thermophilus* ICDH) but not in yeast NAD⁺-specific ICDH (IDH2) or IPMDH from *T. thermophilus* [24]. These results thus suggest that these lysine residues may also be needed for the NADP⁺-binding along with the conserved structures as

discussed above. However, these hypothetical predictions have to be supported by the future studies of site-directed mutagenesis, expression, and biochemical analyses.

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REFERENCES

- 1 Plaut, G. W. E. (1970) *Curr. Top. Cell. Regul.* **2**, 1–27
- 2 Koshland, D. E., Jr., Walsh, K. and LaPorte, D. C. (1985) *Curr. Top. Cell. Regul.* **27**, 13–22
- 3 Burke, W. F., Johnson, R. A. and Reeves, H. C. (1974) *Biochim. Biophys. Acta* **351**, 333–340
- 4 Garnak, M. and Reeves, H. C. (1979) *Science* **203**, 1111–1112
- 5 Nimmo, G. A., Borthwick, A. C., Holms, W. H. and Nimmo, H. G. (1984) *Eur. J. Biochem.* **141**, 401–408
- 6 Dean, A. M., Lee, M. H. I. and Koshland, D. E., Jr. (1989) *J. Biol. Chem.* **264**, 20482–20486
- 7 Walsh, K. and Koshland, D. E., Jr. (1984) *J. Biol. Chem.* **259**, 9646–9654
- 8 Hathaway, J. A. and Atkinson, D. E. (1963) *J. Biol. Chem.* **238**, 2875–2881
- 9 Cohen, P. F. and Colman, R. F. (1972) *Biochemistry* **11**, 1501–1508
- 10 Rutter, G. A. and Denton, R. M. (1989) *Biochem. J.* **263**, 445–452
- 11 Machado, A., Nunes de Castro, I. and Mayer, F. (1975) *Mol. Cell Biochem.* **6**, 93–100
- 12 Stein, A. M., Stein, J. H. and Kirkman, S. K. (1967) *Biochemistry* **6**, 1370–1379
- 13 Pettit, F. H. and Reed, L. J. (1982) *Methods Enzymol.* **89**, 376–386
- 14 Stepp, L. and Reed, L. J. (1985) *J. Biol. Chem.* **260**, 9454–9458
- 15 Lathe, R. (1985) *J. Mol. Biol.* **183**, 1–12
- 16 Davis, L. G., Dibrine, M. D. and Battey, J. F. (1986) in *Basic Methods in Molecular Biology*, Elsevier Science Publishing Co., New York
- 17 Haselbeck, R. J. and McAlister-Henn, L. (1991) *J. Biol. Chem.* **266**, 2339–2345
- 18 Von Heijne, G. (1986) *EMBO J.* **5**, 1335–1342
- 19 Smyth, G. E. and Colman, R. F. (1991) *J. Biol. Chem.* **266**, 14918–14925
- 20 Haselbeck, R. J., Colman, R. F. and McAlister-Henn, L. (1992) *Biochemistry* **31**, 6219–6223
- 21 Thorsness, P. E. and Koshland, D. E., Jr. (1987) *J. Biol. Chem.* **262**, 10422–10425
- 22 Miyazaki, K., Eguchi, H., Yamagishi, A., Wakagi, T. and Oshima, T. (1992) *Appl. Environ. Microbiol.* **58**, 93–98
- 23 Cupp, J. R. and McAlister-Henn, L. (1991) *J. Biol. Chem.* **266**, 22199–22205
- 24 Imada, K., Sato, M., Tanaka, N., Katsube, Y., Matsuura, Y. and Oshima, T. (1991) *J. Mol. Biol.* **222**, 725–738
- 25 Ramachandran, N. and Colman, R. F. (1977) *Biochemistry* **16**, 1564–1572
- 26 Jennings, G. T., Sadleir, J. W. and Stevenson, P. M. (1990) *Biochim. Biophys. Acta* **1034**, 219–227
- 27 Seelig, F. G. and Colman, R. F. (1977) *J. Biol. Chem.* **252**, 3671–3678
- 28 Robinson, J. B., Jr. and Srere, P. A. (1985) *J. Biol. Chem.* **260**, 10800–10805
- 29 Barnes, S. J. and Weitzman, P. D. J. (1986) *FEBS Lett.* **201**, 267–270
- 30 Plaut, G. W. E., Cook, M. and Aogaichi, T. (1983) *Biochim. Biophys. Acta* **760**, 300–308
- 31 Gonzalez-Villasenor, L. I. and Powers, D. A. (1985) *J. Biol. Chem.* **260**, 9106–9113
- 32 Hurley, J. H., Thorsness, P. E., Ramalingam, V., Helmers, N. H., Koshland, D. E., Jr. and Stroud, R. M. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8635–8639
- 33 Rossmann, M. G., Moras, D. and Olsen, K. W. (1974) *Nature (London)* **250**, 194–199
- 34 Hurley, J. H., Dean, A. M., Sohl, J. L., Koshland, D. E., Jr. and Stroud, R. M. (1990) *Science* **249**, 1012–1015
- 35 Wierenga, R. K., De Maeyer, M. C. H. and Hol, W. G. J. (1985) *Biochemistry* **24**, 1346–1357
- 36 Scrutton, N. S., Berry, A. and Perham, R. N. (1990) *Nature (London)* **343**, 38–43