Xenopus Cytosolic Thyroid Hormone-binding Protein (xCTBP)
is Aldehyde Dehydrogenase Catalyzing the Formation of
Retinoic Acid*

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Amino acid sequencing of an internal peptide fragment derived from purified Xenopus cytosolic thyroid hormone-binding protein (xCTBP) demonstrates high similarity to the corresponding sequence of mammalian aldehyde dehydrogenase 1 (ALDH1) (Yamauchi, K., and Tata, J. R. (1994) Eur. J. Biochem. 225, 1105–1112). Here we show that xCTBP was co-purified with ALDH and 3,3',5-triiodo-L-thyronine (T₃) binding activities. By photoaffinity labeling with [¹²⁵I]T₃, a T₃-binding site in the xCTBP was estimated to reside in amino acid residues 93–114, which is distinct from the active site of the enzyme but present in the NAD⁺ binding domain. The amino acid sequences deduced from the two isolated xALDH cDNAs (xALDH-I and xALDH-II) were 94.6% identical to each other and very similar to those of mammalian ALDH enzymes. The two recombinant xALDH proteins exhibit both T₃ binding activity and ALDH activity converting retinal to retinoic acid (RA), which are similar to those of xCTBP. The mRNAs were present abundantly in kidney and intestine of adult female Xenopus. Interestingly, their T₃ binding activities were inhibited by NAD⁺ and NADH but not by NADP⁺ and NADPH, whereas NAD⁺ was required for their ALDH activities. Our results demonstrate that xCTBP is identical to ALDH1 and suggest that this protein might modulate RA synthesis and intracellular level of free T₃.

A major characteristic of 3,3',5-triiodo-L-thyronine (T₃), the active form of thyroid hormone at the cellular level, is the multiplicity of physiological processes. These include such diverse functions as postembryonic and fetal development and postnatal growth in mammals, amphibian metamorphosis, maturation of central nervous system, energy metabolism in homeotherms, and environmental adaptation in poikilotherms (1–4). It is now generally accepted that, at the molecular level, most of these actions of thyroid hormone are initiated by the interaction between thyroid hormone receptor and T₃. Thyroid hormone receptor is a member of a multigene family of nuclear receptors that act as transcription factors in combination with transcriptional co-activators and corepressors and chromatin-modifying factors (5–9). It is, however, not clear as to how T₃ enters the cell and reaches the nucleus and what determines the dynamics of cytoplasm-to-nucleus transfer of the hormone. A key component of this intracellular process is most likely to be the cytosolic thyroid hormone-binding protein (CTBP). Recently, CTBPs have been detected in mammalian and amphibian cells (10–15), an interesting feature of which is that they exhibit different biochemical properties. We have earlier described a CTBP in adult Xenopus liver (xCTBP) (11) which is a 59-kDa protein with a higher affinity for T₃ than l-thyroxine (T₄), T₃ binding being neither Ca²⁺- nor NADPH-dependent, as is the case for Rana and rat CTBPs (12–15).

The physiological actions of retinoic acid (RA) and other retinoids are also considered to be exerted through nuclear retinoid acid receptors (7, 16). There is also good evidence that a large fraction of RA and retinoids is present in the cell bound to cytoplasmic proteins, identified as cytosolic retinoid acid (CRABP) and retinol-(CRBP) binding proteins (16, 17). It has been suggested that these binding proteins may not only determine the intracellular concentration of free ligands but may also act as their transporters into the nucleus. A similar suggestion, based on indirect evidence, has also been made for a mammalian CTBP that has been identified as a monomer of pyruvate kinase subtype M2 (18). Although there are many similarities between CTBP, CRABP, and CRBP, on the one hand, and retinoid acid receptors and thyroid hormone receptors, on the other, a major difference is the multiple types of CTBPs, unlike CRABP and CRBP (17). We have previously reported three types of xCTBPs (19), each with a distinct pattern of expression, which raises the possibility of a tissue-specific role for CTBPs. It therefore became important to characterize CTBPs in greater detail.

A unique feature of xCTBP found in adult liver is that it has a region similar to those of mammalian class I aldehyde dehydrogenase (ALDH1) (aldehyde:NAD⁺ oxidoreductase, EC 1.2.1.3) (11), which is one of the enzymes catalyzing the oxidation of various aliphatic and aromatic aldehydes to the corresponding acids. An important and rather specific activity of ALDH1 is to act as an enzyme catalyzing the synthesis of RA...
from retinal (20–23). It would be highly possible that ALDH1 has a binding activity for hydrophobic signaling molecules including T₃ since human ALDH1 from genital skin fibroblasts displays androgen binding activity (24).

Here we report studies carried out to unambiguously identify xCTBP as ALDH1. Toward this aim, we have cloned two cDNAs encoding ALDH1 from a Xenopus hepatic cDNA library with human ALDH1 cDNA as the probe (25), determined their nucleotide sequences, and examined both the enzyme and T₃ binding activities using the recombinant proteins expressed in Escherichia coli. Deduced amino acid sequences and studies of their activities clearly showed that the two translated products are ALDH1 with T₃ binding activity. The corresponding mRNA were expressed predominantly in the kidney and intestine in adult Xenopus. T₃ binding and ALDH activities of these proteins seem to be expressed alternatively depending on NAD⁺ binding.

**EXPERIMENTAL PROCEDURES**

**General—**Enzymes and chemicals were obtained from the following sources: restriction enzymes from Life Technologies, Inc., New England BioLabs, Inc., Pharmacia Biotech, Takara Shuzo, and Toyobo; [125I]T₃ from NEN Life Science Products; and unlabeled T₃, D-T3, T₄ from ICN Biomedicals Inc; [³²P]deoxyCTP (122 MBq/mmol) was from ICN Biomedicals Inc.; [α-³²P]dCTP (122 MBq/mg; carrier-free) was from NEN Life Science Products; and unlabeled T₃, T₂, T₁, 3',3',5-triiodo-L-thyroacetic acid and all-trans-retinol were from Sigma. Acetaldelyde was obtained from Merck and AG 1-X8 resin from Bio-Rad. Other reagents of molecular biology grade were purchased from Wako Pure Chemicals and ICN Biomedicals. Adult Xenopus hepatic cDNA library in ZAP II λ vector was kindly provided by Dr. A. Kawa-hara, Hiroshima University, Japan. Human ALDH1 cDNA was a gift from Dr. A. Yoshida, Beckman Research Institute of the City of Hope, CA. Protein was determined by the dye binding method with bovine γ-globulin as a standard (26).

**Preparation of Cytosol—**Adult female Xenopus laevis were anesthe-tized by immersing in 0.2% ethyl-3-aminobenzoate methanesulfonic acid salt. Animals were in first perfusion with Barth-X amphibian Ringer (27) containing 0.2 mg/ml heparin and then with ice-cold Barth-X amphibian Ringer alone. Dissected tissue was minced with scissors in Barth-X amphibian Ringer, followed by several washings in the same solution. The minced tissue was homogenized in 4:5 volumes of 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, 1 mM MgCl₂, 1 mM dithiothreitol (DTT), 1 mM benzamidene hydrochloride, and 1 mM phenylmethylsulfonyl fluoride, pH 7.5, and centrifuged (3000 × g; 4 °C) for 10 min. The supernatant was adjusted to 400 × g for 20 min, and 100,500 × g for 60 min, a clear supernatant was obtained and stored in 10% glycerol at −85 °C until its use as a cytosol.

**Purification of Native xCTBP from Liver Cytosol—**xCTBP was puri-fied as described with some modifications (11). All the following procedures were carried out at 4 °C, unless otherwise noted. In brief, solid ammonium sulfate at a final concentration of 1.4 M was added to 30 ml of cytosol, and the precipitate was removed by centrifugation at 12,000 × g for 15 min. More solid ammonium sulfate, at a final concentra-tion of 2.5 M, was added to the supernatant. The precipitate obtained was collected by centrifugation in the same way and dissolved in 2–3 ml of 20 mM sodium phosphate, 0.5 mM DTT, pH 7.5. It was applied to a Cellulose GCL-1000 column (3.0 × 76.5 cm, Seikagaku Co.), which had been equilibrated with the same buffer, and eluted at a flow rate of 0.5 ml/min. The eluates with T₃ binding activity were collected by chromatography on a hydrophobic interaction phenyl 5PW column (7.5 × 75 mm, Tosoh, Tokyo, Japan), equilibriated with 0.5 M ammonium sulfate in 5 mM sodium phosphate and 0.5 mM EDTA, pH 7.0. The proteins were resolved with a 20 ml linear gradient of the buffer to 60% ethylene glycol in 5 mM sodium phosphate, 0.5 mM DTT, pH 7.0, at a flow rate of 0.5 ml/min using a high performance liquid chromatography (HPLC) apparatus (Jasco 851-GI system, Spectroscopic Co.).

**Screening of cDNA Library and Sequence Analysis—**An hepatic cDNA library was screened with ³²P-radioabeled human ALDH1 cDNA (25). The entire sequences of the two cDNAs were determined for both strands by the method of Sanger et al. (28). The computer program, Clustal W (1.60) in DNA Data Bank of Japan was used on multiple sequence alignment and the construction of unrooted tree by the Neigh-bor-joining method (29).

**Northern Blot Analysis—**Total RNA was prepared from them by the acid guanidinum isothiocyanate/phenol/chloroform method (30). Total RNA (15 μg) was electrophoresed on a 1% agarose gel containing 2 M formaldehyde, and the separated RNAs were transferred to a nylon filter. Hybridization and washes were performed under high stringency conditions as described (31). The probe for Xenopus ALDH1 (xALDH1) cDNAs, a 0.3-kbp fragment that contained nt 1708–2014 of xALDH1-1 cDNA, was amplified by polymerase chain reaction (PCR) and labeled with [α-³²P]dCTP. To check the amount of total RNA loaded, 28 S ribosomal RNA hybridization signals on the same filter were estimated as a loading control. Xenopus 28 S ribosomal cDNA was amplified at the nt positions 1–346 by PCR, after the reaction with Mooney murine leukemia virus-reverse transcriptase in the presence of (dT₁₅)₂₀ at 37 °C for 1 h. Autoradiography was done with Kodak XAR5 film with intensifying screen at −85 °C for 1–7 days.

**Expression of Recombinant xALDHs in E. coli—**The coding sequences of xALDH1-I and -II cDNAs, with a NdeI site engineered into the start codon and a BglII site downstream from the stop codon, was prepared by PCR, subcloned into pET15b expression vector (Novagen, Madison, WI), and designated pET15b/xALDH1-II. These plasmids were transformed into E. coli BL21. Bacteria were grown at 37 °C until the absorbance at 600 nm reached 0.5. The temperature was lowered to 24 °C, 0.2 mM isopropyl-1-thio-b-D-galactopyranoside was added, and the culture was held at 24 °C until otherwise noted.

**Purification of Recombinant xALDHs from E. coli—**Bacteria were pelleted by centrifuging (1200 × g), resuspending in 0.3 M NaCl, 50 mM Tris, pH 8.0, 10 mM imidazole, 1 mM lysosome, 1 mM benzamidene hydrochloride, 1 mM phenylmethylsulfon fluoride, and 50 mM 2-mercaptoethanol, and then keeping on ice for 30 min. The cells were disrupted by sonication for 10 s three times on ice at the range 5–10°C and then thawing, and the lysate was centrifuged at 105,000 × g for 40 min at 4 °C. Recombinant proteins with a histidine tag were isolated from the other proteins in the supernatant by a nickel affinity chromatography (1 ml of the resin) (ProBond Resin, Invitrogen, CA), with 0.3 M NaCl, 50 mM Tris, pH 8.0, 250 mM imidazole, after washing with the column with six times column volume of 0.5 M NaCl, 50 mM Tris, pH 8.0, 50 mM imidazole. The purified proteins were stored in 1 ml EDTA, 1 ml DTT, and 10% glycerol at −85 °C until use.

**T₃ Binding Activity—**Cytosolic or recombinant proteins were incubated in 250 μl of 20 mM Tris-Cl, 1 mM DTT, pH 7.5, containing 0.1 nM [¹²⁵I]T₃ in the presence of or absence of 5 μM unlabeled T₃ at 0 °C for 30 min. The [¹²⁵I]T₃ bound to proteins was separated from free [¹²⁵I]T₃ by the Dowex method (11), and these radioactivities were measured in a γ counter (Auto Well Gamma System ARC-2000, Aloka, Japan). The amount of [¹²⁵I]T₃ bound nonspecifically was derived from the radioactivity in the sample incubated with 5 μM unlabeled T₃ and subtracted from amount of the total bound T₃ to give the values for specific binding. The values for the dissociation constant (Kd) and maximum binding capacity were calculated from the Scatchard plot (32).

**Photoaffinity Labeling—**Photoaffinity labeling with underivatized [¹²⁵I]T₃ was carried out as described previously (11). The proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (33) and visualized using Coomassie Brilliant Blue R-250 staining or silver staining. Phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase were used as molecular weight standards. The labeled proteins were detected by autoradiography exposed to x-ray XAR5 film.
(Kodak) at \(-85^\circ C\) for 2–5 days.

**ALDH Activity**—Assay was performed in duplicate or triplicate (values within 10% of the mean) in 100 \(\mu l\) of 50 mM Tris, pH 8.0, 3.3 mM pyrazole, 100 mM KCl, 1 mM DTT, 0.33 mM NAD\(^+\), and various concentrations of substrates by monitoring the formation of NADH (at 340 nm) with aldehydes other than retinal. The reactions were initiated by adding the enzyme. At least six concentrations were used for determining kinetic constants of acetaldehyde, propionaldehyde, and retinal, ranging 1–32 mM, 0.2–16 mM, and 1–32 \(\mu M\), respectively. For RA synthesis, the formation of NADH and retinoic acid (at 340 nm = 6220 ± 39,200 – 22,800) was monitored under dim light (34). In some cases, the formation of RA was monitored at 340 nm by HPLC (35). There are few differences in the values of kinetic parameters obtained from the two methods. Kinetic constants were determined under initial velocity conditions linear with time and protein.

**Western Blot Analysis**—Cytosolic proteins from adult liver and two recombinant xALDH1 proteins were separated by electrophoresis on a 10% gel and silver-stained (Fig. 2A). The amino acid sequence of xALDH1-I showed 94.6% identity to that of xALDH1-II through 502 residues. The two sequences were deduced from the two cDNAs and aligned by Clustal, which showed that the major protein peak also coincided with the peak of ALDH activity. The amino acid sequences of xALDH1-I and -II consisted of both amino acid sequences of xALDH1-I and -II and whose molecular weights were calculated to be 55,020 and 55,215, respectively, which agreed well with 59 kDa for xCTBP estimated by SDS-PAGE. Estimated pl values of xALDH1-I (7.08) and -II proteins (7.44) also agreed well with the measured pl value of xCTBP, 7.0 ± 0.1 (11). The amino acid compositions of the xALDH1-I and -II proteins were aligned using a multiple alignment program (36). The amino acid sequence of xALDH1-I showed 94.6% identity with that of xALDH1-II through 502 residues. The two sequenced primary structures of xCTBP showed high identity to those of human ALDH1 in the two regions selected (see Fig. 2B). Two cDNAs with 2.4 kbp, xALDH1-I and xALDH1-II, which were quite similar to but slightly different from each other, were thus isolated. xALDH1-I was distinguished from xALDH1-II in one of the two internal PstI restriction sites (Fig. 2A). When amino acid sequences were deduced from the two cDNAs and aligned with human ALDH1 sequence (25), it is likely that xALDH1-I cDNA starts at the third nt of the putative ATG codon and ends at nt position 2301 and that xALDH1-II cDNA starts at 21 nt upstream from possible start codon and ends at nt position 2341 (Fig. 2B). For xALDH1-II cDNA, the flanking sequence of the possible start site conformed partially to the Kozak criteria (37). A putative polyadenylation signal was present beginning at nt position 2276 for xALDH1-I cDNA and 2317 for xALDH1-II cDNA, in their 3′-untranslated regions. Deduced amino acid sequences of xALDH1-I and -II consisted of both 502 residues including the start site Met and whose molecular weights were calculated to be 55,020 and 55,215, respectively, which agreed well with 59 kDa for xCTBP estimated by SDS-PAGE. Estimated pl values of xALDH1-I and -II proteins were aligned using a multiple alignment program.
sequences exhibited the highest identity (74–80%) with ALDH1 sequences from various species, as well as the subclass of ALDH1 reported as a retinal dehydrogenase type II, RabDH(II) (38, 39), and the second (71%), third (67–68%), and fourth (65–66%) highest identities with human ALDH6, mammalian ALDH2, and human ALDH5, respectively. The cladogram derived from multiple alignment of amino acid sequences of several classes of ALDHs clearly suggests that the two *Xenopus* proteins belong to the class of ALDH1 (Fig. 3).

As regards functional significance of the above comparisons, these *Xenopus* proteins had all 23 of the strictly conserved residues of the aldehyde dehydrogenase superfamily, expressed in phylogenetically diverse organisms (40). By analogy to the human ALDH1 sequence, Cys(C) (41) and Glu(C) (42) might play catalytically essential roles in *Xenopus* proteins, whereas human ALDH2 enzyme also require the Gin(C) (43).

All three residues were conserved in the two xALDH1 sequences. Three of the amino acids interacted with NAD" in the NAD" binding domain of rat ALDH1 (44) and bovine ALDH2 (45), corresponding to Trp(170), Asn(171), and Glu(197) in the *Xenopus* sequences, and were conserved in xALDH1 as well as mammalian ALDH1 sequences.

To identify whether or not the xCTBP purified from liver cytosol is xALDH1, the amino acid sequences of the two regions in xCTBP determined by direct protein sequencing were compared with those deduced from the two xALDH1 cDNAs. The sequences (K)LDLVERDRLSTM and (M)IDKRAVPFTG-SVEGKLIKEAG were identified to the amino acid positions 92–107 and 239–261 of xALDH1-II, but both were distinct from the corresponding sequences of xALDH1-I at amino acid positions 92 and 256. Thus xCTBP is more likely to be xALDH1-II than xALDH1-I.

**Characteristics of Recombinant xALDH1 Expressed in E. coli**—The xALDH1-I and -II proteins expressed in *E. coli* contain an additional 20 residues of a histidine tag. We purified them to almost single band by a nickel affinity chromatography (Fig. 4A). Approximately 7 mg of the purified proteins were obtained from a 250-ml culture. In SDS-PAGE, the apparent molecular weights of the recombinant xALDH1-I and -II proteins with a histidine tag were estimated to be 60 × 10^3, which was a bit bigger than that of xCTBP in liver cytosol, 59 × 10^3 (Fig. 4B, B and C). The two purified recombinant proteins were specifically photoaffinity labeled with [125I]T3 (Fig. 4B), like the xCTBP purified from liver cytosol (see inset in Fig. 1). The photoaffinity labeling of xALDH1-II was more strongly inhibited by 5 μM unlabeled T3 than that of xALDH1-I. Polyclonal antibody to the peptide of xCTBP recognized both recombinant proteins as well as xCTBP in adult liver cytosol (Fig. 4C).

ALDH activity was found in the two recombinant proteins expressed from the xALDH1-I and -II cDNAs with all the substrates examined. The _Km_ values for acetaldehyde, propionaldehyde, retinal, and NAD" are summarized in Table I. Both proteins showed the lowest _Km_ values for retinal (6.9 ± 0.5 μM in xALDH1-I and 4.2 ± 0.2 μM in xALDH1-II) (Fig. 5, B and C); the _Km_ values for propionaldehyde (0.45 ± 0.13 mM in xALDH1-I and 0.32 ± 0.14 mM in xALDH1-II) and acetaldehyde (3.2 ± 0.4 mM in xALDH1-I and 1.7 ± 0.2 mM in xALDH1-II) were 2 and 3 orders of magnitude higher than those for retinal. _V_max_ values of the two proteins for acetaldehyde (0.40 ± 0.12 in xALDH1-I and 0.14 ± 0.02 μmol/min/mg in xALDH1-II) were similar to those for propionaldehyde (0.31 ± 0.07 in xALDH1-I and 0.12 ± 0.01 μmol/min/mg in xALDH1-II) and were 1 order of magnitude higher than those for retinal (0.062 ± 0.005 in xALDH1-I and 0.045 ± 0.003 μmol/min/mg in xALDH1-II). The _V_max_/_Km_ values indicate that substrate preference of the two xALDH1 proteins is the following order: retinal > propionaldehyde > acetaldehyde. The kinetics for NAD" were characterized by _Km_ of 38 ± 3 and 9.2 ± 1.8 μM, and _V_max_ of 0.44 ± 0.10 and 0.18 ± 0.03 μmol/min/mg, for xALDH1-I and -II, respectively, at the concentration of NAD" ranging from 6 to 120 μM. Compared with the kinetics of the purified xCTBP, the _Km_ of *Xenopus* recombinant proteins exhibited values 3–5 times higher, although their _V_max_ value was very similar. The order of substrate specificity of the *Xenopus* enzymes was in agreement with that of mammalian ALDH1 enzymes (38, 46, 47) but quite distinct from other classes of mammalian ALDHs (48–51). Positive cooperativity showing allosteric kinetics could only be detected when the ALDH activities of the xALDH1-I and -II proteins were examined with various concentration of retinal (Hill coefficients, _n_ = 1.8 ± 0.4 and 2.7 ± 0.6, respectively) (Fig. 5, B and C) but were undetectable with acetaldehyde and propionaldehyde as substrates.

**Relationship between T3 Binding and ALDH Activities on xCTBP/xALDH1**—To confirm whether or not the two recombinant xALDH1 proteins are dual-functional proteins, T3 bind-
Xenopus CTBP with Retinal Dehydrogenase Activity

**FIG. 2.** Restriction maps, nucleotide sequences, and deduced amino acid sequences of two xALDH1 cDNAs. A. open boxes indicate the coding regions, and solid lines indicate the non-coding regions. The letters B, E, H, Ps, and Pvu represent BamHI, EcoRI, HindIII, PstI, and PvuII.
ing activity was also examined. The two recombinant proteins bound specifically T₃ (insets in Fig. 6). Their binding specificities were T₃, D-T₃, T₄, 3,3',5-triiodo-L-thyroacetic acid, which was very similar to that of the xCTBP in adult liver cytosol (11). [125I]T₃ binding to xALDH1-II was more strongly inhibited by 320 nM unlabeled T₃ than that to xALDH1-I, which was in good agreement with the results of the photoaffinity labeling (Fig. 4B). The results of Scatchard analysis of the xALDH1-I and -II proteins shown in Fig. 6 and their K_d values for T₃ binding are compared with those of the xCTBP from liver cytosol determined previously (11) (Table II). The K_d values, 142 ± 0 and 48.0 ± 7.2 nM for the recombinant xALDH1-I and -II proteins, were 16 and 5 times higher than that for the xCTBP purified from liver cytosol (9 nM), respectively. As the recombinant xALDH1 proteins required 10⁻⁵ to 10⁻⁴ M NAD⁺ to display ALDH activity (Table I), the effects of NAD⁺ on T₃ binding by the two recombinant proteins were examined. NAD⁺ can inhibit the T₃ binding to the two xALDH1 proteins in a dose-dependent manner. The concentrations of NAD⁺ necessary to inhibit 50% of specific T₃ binding were about 100 and 43 μM for xALDH1-I and xALDH1-II, respectively (not shown). These findings suggest that xCTBP/xALDH1 expresses alternatively T₃ binding and ALDH activities dependent on NAD⁺ binding. Therefore, we next examined the effect of four coenzymes, including NAD⁺ and NADH, on the T₃ binding to the recombinant xALDH1 proteins. NAD⁺ and NADH both inhibited T₃ binding activity by 47 and 27% for xALDH1-I and 23 and 18% for xALDH1-II, respectively, at the concentration of 0.2 mM, but restriction sites. B, first lines, nucleotide sequences of xALDH1-I cDNA; second lines, amino acid sequence deduced from xALDH1-I cDNA sequence; third lines, amino acid sequence deduced from xALDH1-II cDNA sequence; fourth lines, nucleotide sequences of xALDH1-II cDNA. The AATAAA polyadenylation signal close to the 3' end of the cDNAs is written in bold letters. The two underlined peptides show the regions corresponding to the sequences determined by a direct protein sequencing. The N-terminal region was determined using the peptide derived from the liver xCTBP after digestion with lysyl endopeptidase, and the C-terminal one was determined using the peptide after treatment with CNBr (11). Residues essential for catalytic activity (41–43), Trp¹⁷⁰, Asn¹⁷³, Glu²⁵⁷, Glu²⁷⁰, Cys²⁸⁳, and Gln⁴⁸⁹, are underlined. Amino acid residues of xALDH1-I are represented by dots where they are identical to the xALDH1-II sequence.
NADP⁺ and NADPH failed to exert the effect at the same concentration (Fig. 7).

Expression of xCTBP/xALDH1 in Different Tissues—In all tissues examined, a single band of mRNA was detected in a size between 28 S and 18 S ribosomal RNAs. The accumulation of xCTBP/xALDH1 transcripts was particularly strong in kidney and intestine, with smaller amounts found in liver and stomach. These transcripts were expressed at very low levels in heart and skeletal muscles (Fig. 8).

DISCUSSION

From the following five lines of evidence, we conclude that xCTBP is xALDH1. First, the xCTBP purified from liver cytosol displayed ALDH activity (Fig. 1 and Table I), and its enzymatic properties agreed with those of mammalian ALDH1. Second, the recombinant proteins expressed from two xALDH1 cDNAs in E. coli also showed both ALDH and T₃ binding activities (Figs. 4–7), which were photoaffinity labeled with [¹²⁵I]T₃ in the absence (lanes 1, 2, and 4) or presence (lanes 3 and 5) of 5 μM unlabeled T₃. A 10% gel was run, followed by autoradiography. C, immunoreactivity of two recombinant xALDH1 proteins with a polyclonal antibody against a peptide of xCTBP. Cytosolic proteins (93 μg) from adult liver (lane 1) and the purified recombinant proteins (each 8 μg), xALDH1-I (lane 2) and xALDH1-II (lane 3), were immunoblotted after SDS-PAGE as described under “Experimental Procedures.” Arrowheads indicate recombinant xALDH1 proteins with a histidine tag.

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FIG. 4. Purification of two recombinant xALDH1 proteins and comparison of their T₃ binding activities and immunoreactivities with those of xCTBP in liver cytosol. A, purification of two recombinant xALDH1 proteins. Bacteria harboring pET15b/xALDH1-I (lanes 1–4) or pET15b/xALDH1-II (lanes 5–8) were cultured in the presence of 0.2 mM isopropyl-β-D-thiogalactopyranoside for 24 h at 24 °C. These extracts were mixed with a nickel-bound resin, and the mixtures were packed into a disposable mini-column (7 × 23 mm). After washing the column, xALDH1-I and -II were eluted as described under “Experimental Procedures.” Lanes 1 and 5, bacterial extract; lanes 2 and 6, flow-through fraction; lanes 3 and 7, eluates with 80 mM imidazole buffer; lanes 4 and 8, eluates with 250 mM imidazole buffer. B, photoaffinity labeling of xCTBP and xALDH1 with [¹²⁵I]T₃. Cytosolic proteins (65 μg) from adult liver (lane 1) and the purified recombinant proteins (each 5.6 μg), xALDH1-I (lanes 2 and 3) and xALDH1-II (lanes 4 and 5), were photoaffinity labeled with [¹²⁵I]T₃ in the absence (lanes 1, 2, and 4) or presence (lanes 3 and 5) of 5 μM unlabeled T₃. A 10% gel was run, followed by autoradiography. C, immunoreactivity of two recombinant xALDH1 proteins with a polyclonal antibody against a peptide of xCTBP. Cytosolic proteins (93 μg) from adult liver (lane 1) and the purified recombinant proteins (each 8 μg), xALDH1-I (lane 2) and xALDH1-II (lane 3), were immunoblotted after SDS-PAGE as described under “Experimental Procedures.” Arrowheads indicate recombinant xALDH1 proteins with a histidine tag.

FIG. 5. Rates of RA synthesis from retinal catalyzed by xCTBP purified from adult liver and by recombinant xALDH1 enzymes versus substrate concentrations. The rate of RA synthesis was measured in the presence of the xCTBP (A), xALDH1-I (B), or -II (C) enzymes. These reactions were performed at 24 °C with 1.85 μg of xCTBP or 5 μg of xALDH1-I and -II enzymes. The insets depict Hill plots. Each value is the mean of triplicate determinations.
Xenopus CTBP with Retinal Dehydrogenase Activity

xCTBP and the two recombinant xALDH1 proteins. Since retinal is a preferred substrate for xALDH1, our conclusion raises the possibility that xCTBP/xALDH1 can modulate actions of RA and T3 via their nuclear receptors by regulating RA synthesis and intracellular levels of free T3.

As regards specifically the Xenopus proteins, the Km and Kd values of the recombinant xALDH1 proteins were several times higher than those of xCTBP from liver cytosol. The lower affinities of the recombinant xALDH1 proteins for the substrates and T3 might be due to the presence of the histidine tag at their N termini, which may be a contributory factor for the variation.

**FIG. 6. Scatchard analysis of T3 binding to the xALDH1 proteins.** The purified recombinant xALDH1-I (24.8 μg, A) and -II (12.8 μg, B) were incubated with 0.1 nM [125I]T3 at various concentrations of unlabeled T3 for 30 min at 0 °C in a final volume of 250 μl of 20 mM Tris-HCl, 1 mM DTT, pH 7.5. B/F, bound/free ratio. Each value indicates the mean of triplicate determinations. The insets illustrate the effect of four competitors (320 nM) on [125I]T3 binding to the purified recombinant xALDH1-I (A) and xALDH1-II (B). Triac, 3,3',5-triiodo-L-thyroacetic acid. *, p < 0.01; **, p < 0.001, compare [125I]T3 binding in the presence of the competitor with that in the absence of the competitor. Nonspecific binding was subtracted from total binding. Each value indicates the mean ± S.E. of triplicate determinations.

| | Kd (nM) | MBC (pmol T3/mg protein) |
|---|---|---|
| xCTBP (3) | 9.6 | 152 |
| xALDH1-I (3) | 142 ± 0a | 268 ± 29 |
| xALDH1-II (3) | 48 ± 7.2 | 260 ± 14 |

*T3 binding to xCTBP and xALDH enzymes*

The Kd and maximum binding capacity (MBC) values for xCTBP are cited (4). The number of determinations is shown in parentheses.

*a p < 0.01, compared kinetic constants of xALDH1-I with those of xALDH1-II.*
in the synthesis of RA from retinal. It is worth noticing that the ability to convert to RA is found in many ALDH1 proteins, including RalDH(II) as a subtype, from various vertebrate species (indicated by asterisks in Fig. 3). In view of the involvement of RA in many developmental processes, it would be valuable to survey the presence of subtypes of ALDH1 and to examine the expression patterns of a group of xALDH1 proteins during early embryogenesis and limb formation in Xenopus.

Sequence comparison of ALDHs depicted in the cladogram (Fig. 3) shows that the residues Glu270, Cys304, and Gln489, which are thought to be involved in the catalytic role of ALDH enzymes (41–43), were highly conserved in Xenopus sequences. The region participating in T3 binding by xCTBP/xALDH1 is located at amino acid positions 92–107. The corresponding region is present away from the catalytic domain but in the NAD+ binding domain in bovine ALDH2 (45). Although this region does not seem to interact directly with NAD+, the structural basis for the effects of NAD+ on T3 binding will probably require a structure for ALDH1.

Both recombinant xALDH1 enzymes can catalyze the dehydrogenation of acetaldehyde, propionaldehyde, and retinal (Table I). The substrate specificity estimated as a \( V_{\text{max}}/K_{\text{m}} \) is the highest for retinal, this value being 7–12- and 50–90-fold higher than those for propionaldehyde and acetaldehyde, respectively, whereas for xCTBP, the \( V_{\text{max}}/K_{\text{m}} \) for retinal is 30-fold higher than that for acetaldehyde. Interestingly, allosteric kinetics showing positive cooperativity was observed for the recombinant xALDH1 enzymes and the xCTBP with the Hill coefficient of 1.8–2.7 and 2.5, respectively, when retinal was used as the substrate (Table I). A similar observation was made for a rat RalDH(I), with Hill coefficient of 1.4–1.8, by Napoli’s group (52–55). Napoli’s group also reported that retinal associated with cytosolic retinoid-binding proteins (CRABP and CRBP) could be important for its recognition as a substrate by the enzyme, since CRABP stimulated the production of RA and CRBP suppressed it (53). This interesting finding suggests that it would be useful in future studies to determine a similar effect of retinoid-binding proteins on the enzyme activities of xCTBP/xALDH1.

The two xALDH1 enzymes reported here are quite similar to each other as regards their the primary sequences, substrate specificities, and T3 binding properties (Tables I and II, and Fig. 2). Probably the presence of two genes is most likely due to the tetraploidy of the Xenopus genome (56). For this reason we used the 3’ non-coding region of the xALDH1-I cDNA as a probe for Northern analysis, since it would recognize both xCTBP/xALDH1 transcripts but not those of the other members of ALDH superfamily. The relatively high amounts of xALDH1 mRNAs, migrating as a single band with a mobility intermediate to those of ribosomal 18 S and 28 S RNAs in adult Xenopus kidney and intestine (Fig. 8), are compatible with the finding for rat kidney ALDH1 (57). The level in liver was not so high, although T3 binding activity was found almost exclusively in liver (19). This discrepancy suggests the possibility of post-transcriptional or -translational regulation, or some other form of modulation of T3 binding activity of xCTBP.
hormonal activities could bind to rabbit ALDH. Although very recent study indicated that human liver ALDH1 and ALDH2 both could bind T3 and 3',3',5-triiodo-L-thyroacetic acid, their $K_D$ values were micromolar ranges (58). It would therefore be interesting to determine whether or not the same or other hydrophobic signaling molecules bind to xCTBP/xALDH1.

Among other studies on CTBPs, there are those reporting an enzyme-linked CTBP, which is a monomeric form of pyruvate kinase subtype M2 and described high levels of its transcript in Xenopus tadpole tail just before metamorphic climax, in hindlimb during the progression of metamorphosis, whereas relatively low levels were detected in the intestine during metamorphosis. However, it is uncertain whether or not the monomer of pyruvate kinase subtype M2 functions as a CTBP in Xenopus as in the human cell lines. Our earlier studies have shown that xALDH1 is the predominant CTBP in adult Xenopus (11, 19). It is worth pointing out that there is no similarity of primary sequence between the monomer of the pyruvate kinase subtype M2 and xCTBP/xALDH1. Another type of NADPH-activated CTBPs has been reported in rat kidney (13), liver (14), and brain (15), comprising molecular species with different molecular weight values. For example, kidney CTBP is a monomer of 58 kDa, liver CTBP is a homodimer with 76 kDa, and rat brain CTBP is a 58-kDa protein. $T_3$ binding activity of all of these CTBPs is strongly activated by NADPH and slightly by NADH, but not by NADP$^+$, whereas NAD$^+$ has no effect on the $T_3$ binding activity of the rat CTBPs (13, 14). A very recent study indicated that human kidney CTBP is a 38-kDa protein, which is homologous to karanaroo $\mu$ crystallin (60). On the other hand, $T_3$ binding activity of xCTBP is inhibited by NAD$^+$ and NADH, and neither NADPH nor NADP$^+$ is effective. If the total concentration of cytoplasmic NAD$^+$ plus NADH, which ranges $10^{-4}$ M in mammalian cells (61), changes in similar ranges in Xenopus cells, it would be one of the important factors modulating $T_3$ binding to xCTBP/xALDH1. These observations and our present results show that xCTBP might be a novel type of CTBP.

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