Avian Alcohol Dehydrogenase. Characterization of the Quail Enzyme, Functional Interpretations, and Relationships to the Different Classes of Mammalian Alcohol Dehydrogenase†

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ABSTRACT: The primary structure of the major quail liver alcohol dehydrogenase was determined. It is a long-chain, zinc-containing alcohol dehydrogenase of the type occurring also in mammals and hence allows judgement of the gene duplications giving rise to the classes of the human alcohol dehydrogenase system. The avian form is most closely related to the class I mammalian enzyme (72–75% residue identity), least related to class II (60% identity), and intermediately related to class III (64–65% identity). This pattern distinguishes the mammalian enzyme classes and separates classes I and II in particular. In addition to the generally larger similarities with class I, the avian enzyme exhibits certain residue patterns otherwise typical of the other classes, including an extra Trp residue, present in both class II and III but not in class I, with a corresponding increase in the UV absorbancy. The avian enzyme further shows that a Gly residue at position 260 previously considered strictly conserved in alcohol dehydrogenases can be exchanged with Lys. However, zinc-binding residues, coenzyme-binding residues, and to a large extent substrate-binding residues are unchanged in the avian enzyme, suggesting its functional properties to be related to those of the class I mammalian alcohol dehydrogenases. In contrast, the areas of subunit interactions in the dimers differ substantially. These results show that (a) the vertebrate enzyme classes are of distant origin, (b) the submammalian enzyme exhibits partly mixed properties in relation to the classes, and (c) the three mammalian enzyme classes are not as equidistantly related as initially apparent but suggest origins from two sublevels.

Alcohol dehydrogenases of at least three different enzyme types exist: (a) long-chain, zinc-containing alcohol dehydrogenases, including the traditional liver enzyme (Jörnvall et al., 1987b); (b) short-chain, nonmetalloenzymes, including alcohol dehydrogenases of insects and further enzymes of bacteria (Jörnvall et al., 1981, 1984) and mammals (Krook et al., 1990); and (c) iron-activated alcohol dehydrogenases of certain bacteria and yeasts (Neale et al., 1986; Williamson & Paquin, 1987). Although a common coenzyme-binding structure has been established for these forms (Thatcher & Sawyer, 1980; Jörnvall et al., 1984), relationships are difficult to trace in detail and do not appear to involve the entire chains of these different groups.

The zinc-containing, long-chain alcohol dehydrogenase group has evolved into a family of at least four different proteins and many different sublines, as a result of gene duplications at three separate levels (Jörnvall et al., 1987a). Beside alcohol dehydrogenase, the proteins are sorbitol dehydrogenase (Jörnvall et al., 1981), threonine dehydrogenase (Aronson et al., 1989), and ζ-crystallin (Borrås et al., 1989). The extensive differences in functional properties have been interpreted in terms of structure for each of the lines (Eklund et al., 1985, 1987, 1990; Aronson et al., 1989; Borrås et al., 1989). The mammalian line has been further subdivided into three classes (I, II, and III) (Vallee & Bazzone, 1983), one of which, III, has recently been shown to be identical with glutathione-dependent formaldehyde dehydrogenase (Koivusalo et al., 1989). The structural properties of all these classes are known (Jörnvall et al., 1987a; Kaiser et al., 1988, 1989).

To date, information obtained on vertebrate alcohol dehydrogenases has been confined to the mammalian forms of the enzyme. One exception has been a limited work on a chicken liver alcohol dehydrogenase, which suggested the presence of structures in the avian enzyme characteristic of class I enzymes (von Bahr-Lindström et al., 1978). The avian enzyme analyzed in the present study was obtained from quail liver and also exhibited properties related to those of a class I enzyme (Nussrallah et al., 1989). However, structural properties, charges, and enzyme specificities now show that this enzyme exhibits, in addition, some features apparently typical of class III enzymes. These features may prove promising for tracing the origins and functional roles of the mammalian alcohol dehydrogenases.

Materials and Methods

Alcohol Dehydrogenase. The quail enzyme now analyzed was purified from pooled liver samples of *Coturnix japonica* and represents the form of the enzyme identified as ADH-3 (Nussrallah et al., 1989). The pure protein (in two separate batches of 30–35 nmol) was dissolved in 6 M guanidine hydrochloride, 0.4 M Tris-HCl, and 2 mM EDTA, pH 8.1, reduced with dithiothreitol (5 μmol/10 mg of protein; 37 °C;

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2 h) and carboxymethylated at 37 °C for 1 h with neutralized ^{14}C-labeled iodoacetic acid [(Amersham) diluted to 2.400 cpm/nmol]. Reagents and buffers were removed by dialysis against 1 mM HCl.

Peptides. One-half of the carboxymethylated protein was dissolved in concentrated formic acid, diluted to 70% formic acid, and cleaved with CNBr (100 mg/mL) at room temperature for 24 h. Reagents were removed by rotary evaporation and the digest was prefractionated by chromotography on Sephadex G-50 in 30% acetic acid, followed by a step of reverse-phase high-performance liquid chromatography as described (Jeffery et al., 1984). Before this second purification step, fractions with large peptides were submitted to redigestions with Glu-specific protease from *Staphylococcus aureus* (Miles) or Lys-specific protease from *Achromobacter lyticus* (Waco Chemicals, Neuss, FRG) as described (Kaiser et al., 1988).

The other batch of the carboxymethylated enzyme was cleaved directly with a Lys-specific protease from *Lysobacter enzymogenes* (Boehringer-Mannheim) at an enzyme to protein ratio of 1:50 (37 °C, 4 h). For this digestion, the protein was first solubilized in 9 M urea and then diluted with 0.1 M ammonium bicarbonate, pH 8.1, to a final concentration of 0.9 M urea in this buffer. This digest was fractionated directly by reverse-phase high-performance liquid chromatography on Pharmacia TSK ODS-120T (Kaiser et al., 1988).

Analysis. Total compositions were determined after hydrolysis for 24 h at 110 °C in evacuated tubes with 6 M HCl/0.5% phenol. For analysis down to 50 pmol, a ninhydrin-based amino acid analyzer (Beckman 121M) was utilized, whereas high-performance liquid chromatography of the phenylthiocarbamyl derivatives (Bergman et al., 1986) was utilized for peptides recovered in low yield after redigestions. For sequence analysis, peptides were degraded with Applied Biosystems 470A gas-phase sequencers, utilizing for phenylthiohydantoin identification either an 120A on-line analyzer or a separate high-performance liquid chromatograph (Hewlett-Packard 1090) as described (Kaiser et al., 1988).

Peptides are referred to by a letter-digit nomenclature, the letter indicating the original peptide (B for CNBr; T for trypsin; K for Lys-specific protease; E for Glu-specific protease) and the subsequent digit the relative order of the peptides in the original protein chain. For peptides generated by redigestions, all cleavages used are shown by additional letter-digits given in the same order as the proteolytic fragmentations were employed.

Results

Primary Structure. The amino acid sequence of quail liver alcohol dehydrogenase (ADH-3 form) was determined by peptide analysis, utilizing separate cleavages of the carboxymethylated enzyme with Lys-specific protease and CNBr. CNBr fragments were purified by a two-step procedure utilizing exclusion chromatography and reverse phase high-performance liquid chromatography or, for the largest fragments, were used directly for redigestions to produce smaller peptides suitable for purification and analysis. The peptides from the Lys-specific cleavage were fractionated by high-performance liquid chromatography. The analytical steps and major fractionation results are shown in Figure 1. The amino acid sequence deduced is given in Figure 2.

The sequence results are supported by total compositions from acid hydrolysis, as shown for relevant peptides in a supplement table and for the whole protein in Table I. Fragment positions, extent of analysis, and purification patterns are evident from Figures 1 and 2 and are not further described below, except where special problems were encountered.

Table I: Total Composition of the Quail Protein As Determined by Acid Hydrolysis and Sum of Sequence Analysis[a]

residue	acid hydrolysis		sum of sequence
	this work	Nussrallah et al., 1989	
Cys	8.6	11.7	13
Asp } Asn }	21.6	18.8	{ 15 / 7
Thr	29.2	28.7	28
Ser	23.5	28.2	23
Glu } Gln }	25.7	29.4	{ 23 / 3
Pro	20.5	20.8	19
Gly	36.0	44.0	34
Ala	32.7	36.3	35
Val	32.6	36.1	38
Met	5.7	5.0	7
Ile	19.8	20.9	24
Leu	27.0	24.9	28
Tyr	6.5	5.9	6
Phe	17.3	16.9	17
Trp	+	3.0	3
Lys	35.8	33.8	34
His	9.3	8.3	9
Arg	9.5	12.1	9
Sum			375

[a] A previous preparation (Nussrallah et al., 1989) shows deviations in single residues, mainly high Gly, presumably indicating free amino acid contaminants in that preparation.

One segment inaccessible to direct analysis was the N-terminal region, which is blocked by acylation as in other alcohol dehydrogeneases (Fairwell et al., 1987). It was analyzed by limited acid hydrolysis of peptide K1 (Figure 2) with 10 M HCl for 5 h at room temperature to give deacylation and partial peptide fragmentation. The product thus obtained was submitted to direct sequence analysis, showing the deblocked pentapeptide and lower amounts of constituent di- and tripeptides. The N-terminally blocked CNBr fragment B1 was obtained by a cleavage after Trp-15 (Figure 2), which is a well-known secondary specificity for CNBr (Ozols et al., 1977), also encountered with other alcohol dehydrogenases (Kaiser et al., 1988). Similarly, cleavage was found at remaining tryptophan residues of the quail protein, explaining the presence of peptides B9, B10, and B11 (Figure 2), which, however, were also recovered uncleaved (B8, Figure 2). Formally, the order of peptides K2/K3 and the overlap connecting K7/K8 were not proven by sequence degradations. However, compositions as well as recoveries of secondary fragments upon redigestions of peptides B1 and B3 (Figure 2) show that no intervening peptides were missed at these positions. Furthermore, these structures are consistent with those from other alcohol dehydrogenases.

The C-terminal segment was proven to end with Leu (Figure 2) by the results of sequence degradations and total compositions of both B12 and K31 (supplemental table) and by the presence of C-terminal Leu in the two peptides, neither of which are likely otherwise to produce such a C-terminus from the specificity of cleavage.

Finally, special problems were encountered in the analysis of internal hydrophobic areas covered by CNBr fragments B4–B7. A large part of B4 was inaccessible to direct analysis and was recovered only in low yield from the Lys-specific cleavage (peptides K11–K15, Figure 2) because of poor solubility of the hydrophobic segment. Similarly, yields of peptides B5–B7 were low because of nonstoichiometric cleavages of the Met–Thr bonds at positions 258–259 and 276–277. Material sufficient for analysis of fragments B4–B7

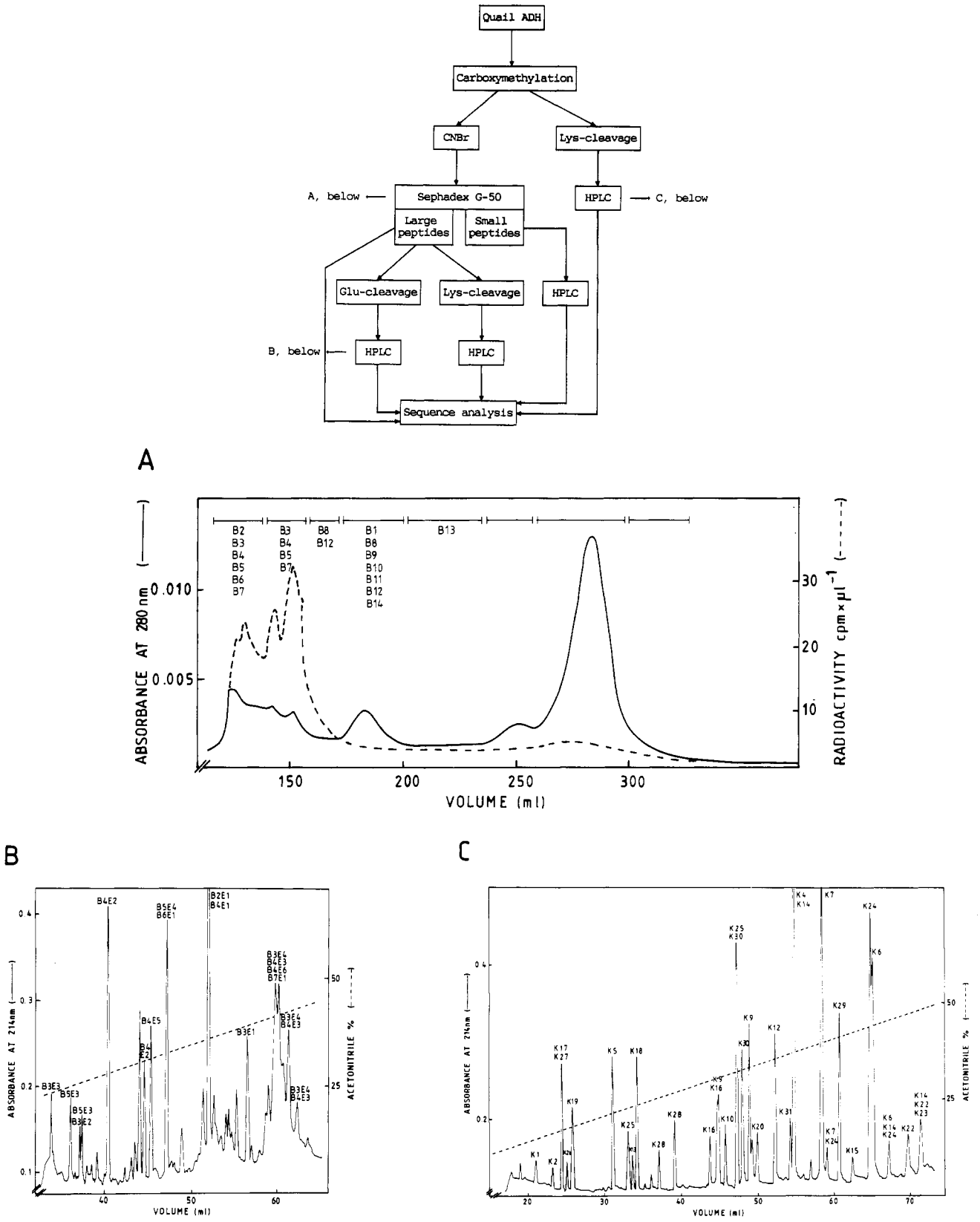

FIGURE 1: Flow scheme illustrating the analytical steps and the patterns of the major fractionation steps.

was therefore obtained by redigestions utilizing the Glu-specific protease. In order to increase yields of the secondary peptides, the parent fraction from the initial separation of the CNBr fragments was digested directly after the Sephadex chromatography step (Figure 1), without high-performance liquid chromatography purification until after the cleavage.

The structure deduced contains one residue more, 375, than the class I mammalian liver alcohol dehydrogenase (Jörnvall et al., 1987b) because of an extra residue in the middle part of the protein chain (after position 118; Figure 3). Thus, in relation to the class I mammalian enzyme, the quail enzyme has an insertion of one Ser residue before position 119.

Comparison with Other Alcohol Dehydrogenase Structures. A comparison of the avian alcohol dehydrogenase structure obtained here and the three classes of mammalian alcohol dehydrogenase (Figure 3) shows that the major quail enzyme

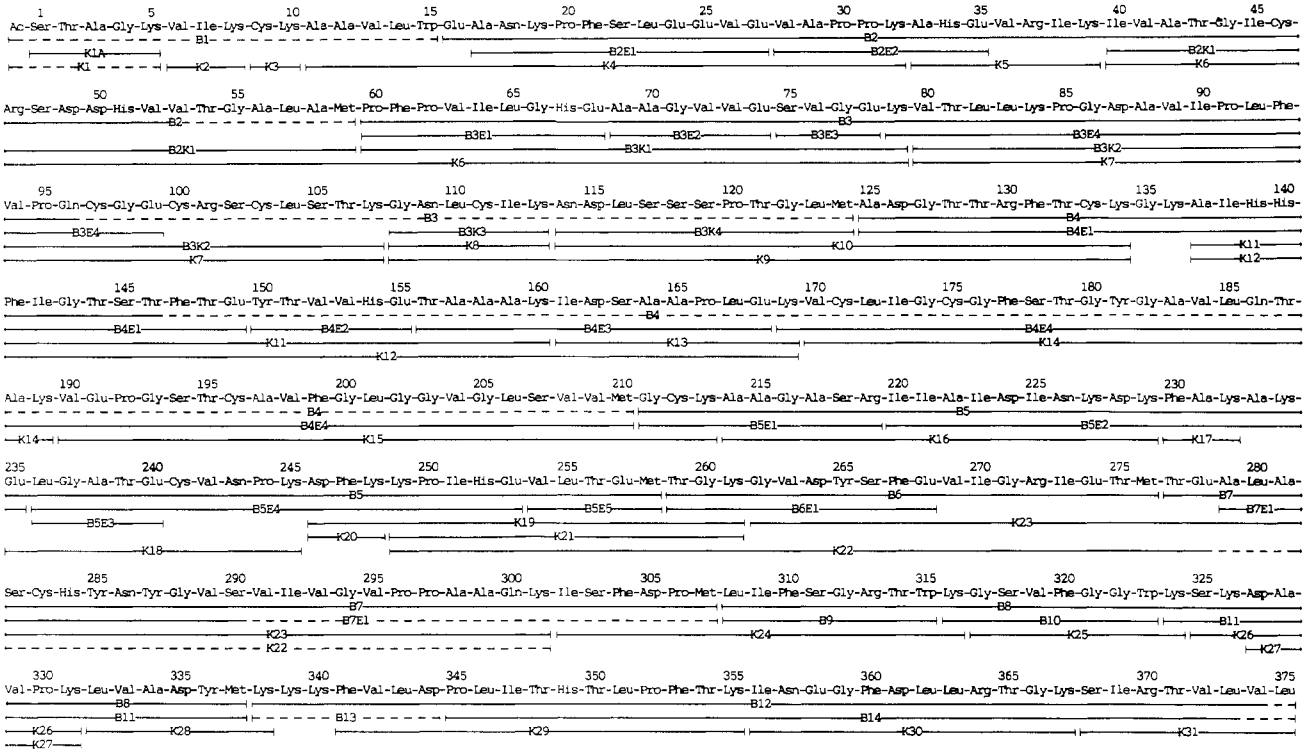

FIGURE 2: Amino acid sequence of the major quail liver enzyme, and positions of all peptides analyzed. Peptide designations show the proteolytic agents used for peptide generation and the order of peptides as explained in the Materials and Methods section. Solid lines indicate parts of each peptide analyzed by sequencer degradation; dashed lines indicate remaining regions analyzed by total composition only.

is most closely related to the class I type (72–75% residue identity, depending on species) and most distantly related to the class II type (60%), with intermediate values for the relationship to class III (64–65%). Regarding the mammalian enzyme classes, these results are of particular interest in relation to three aspects—origin of the classes, origin of the isozymes within the traditional class I type, and time of class separation.

Previously, characterizations of the classes for the mammalian enzymes then available (Jörnvall et al., 1987; Kaiser et al., 1988, 1989; Julià et al., 1988) showed that the three classes resemble separate enzymes but suggested them to be roughly equidistantly related. The combined avian/mammalian data now suggest an evolution of the enzyme classes at two different sublevels, with an initial splitting of two lines and a later separation of a third (Figure 4). Definite class assignments to the lines cannot yet be traced with certainty since details are dependent on the evolutionary rates for the three classes, which differ greatly between classes I and III (Kaiser et al., 1989) but are still unknown for class II. However, the different relationships of the avian enzyme to the classes (Figure 4C) suggest that classes I and II did not coevolve. Hence, the likely line assignments appear to be the two alternatives (A, B) in Figure 4, with lines to present-day classes I or II initially coevolving with that of present-day class III. In any event, a two-level arrangement is suggested, and functionally this scheme outlines a separate role especially for class II versus that of the liver enzyme of class I. A similar conclusion about separate lineages has been reached from studies of an eye lens crystallin protein distantly related to the alcohol dehydrogenases (Borrás et al., 1989). Furthermore, the greater similarity of the major quail enzyme to the mammalian class I enzyme than to the enzymes of remaining classes shows this class to be the one of common abundance in birds and mammals. Different enzyme functions for the three mammalian classes are also consistent with nonidentical evolutionary properties for the proteins (Kaiser et al., 1989) and a separate additional enzyme activity for one of the classes (Koivusalo et al., 1989).

In spite of the general resemblance to class I, it should be noted that the avian enzyme also has several features otherwise typical of the class II and/or III mammalian enzymes. The latter include a tryptophan residue at position 322 and an extra residue before position 119 (Figure 3). Thus, not only in origin (Figure 4), but also in structural features typical of mammalian class-distinguishing properties (Kaiser et al., 1989), the avian enzyme establishes partly mixed interconnections.

Regarding the intraclass mammalian isozymes, the quail enzyme reveals no greatly different relationships to any of the α-, β-, and γ-subunit types. However, the differences are smaller toward the horse E and human γ subunits than toward the other class I subunits. Therefore, the apparently closer relationship of the horse E subunit with the human γ subunit than with the other human class I subunit types (Bühler et al., 1984) is now confirmed also in relation to more distant forms.

The determination of the amino acid replacements of the avian enzyme provides a possibility to estimate the time for the gene duplications leading to the class distinctions (Figure 4). Thus, the quail/mammalian enzyme differences are found to be similar to or in one case even smaller than the mammalian interclass differences, as revealed by the identity levels (Figure 4), suggesting the class origin to be an early event, probably before the mammalian radiation and possibly before the avian/mammalian separation, at least for the first duplication leading to the class separations. If so, one would predict the occurrence of additional alcohol dehydrogenase classes also in birds. Although such enzymes have still not been characterized, their presence appears compatible with the multiplicites reported (Nussrallah et al., 1989). Thus, it would suggest that at least some class multiplicity may be a vertebrate rather than just a mammalian property. Finally,

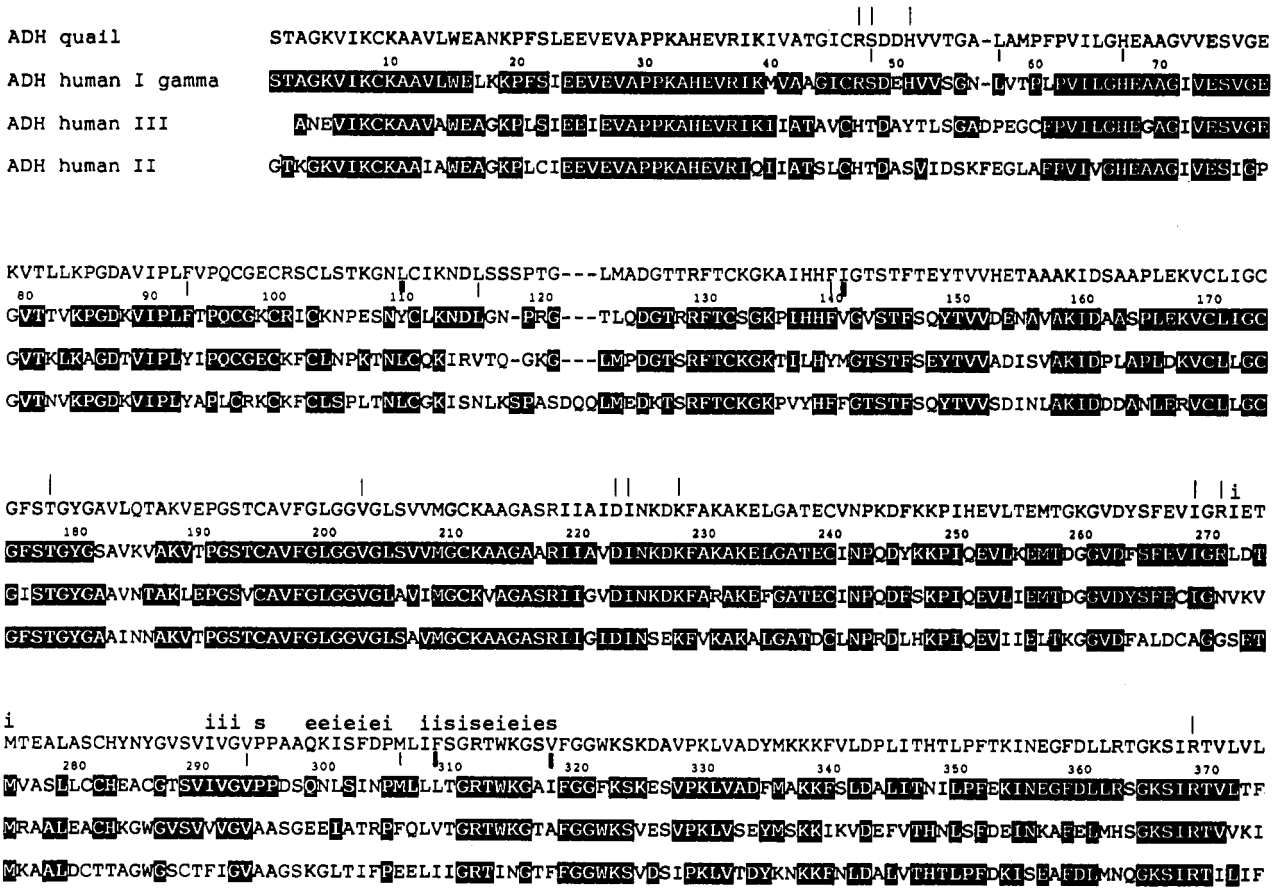

FIGURE 3: Alignment of the major quail liver alcohol dehydrogenase (top) with the three classes of mammalian alcohol dehydrogenase, represented by the γ subunit of the human enzyme of class I, the π subunit of class II, and the χ subunit of class III. Positions deduced to correspond to three types of functional interactions are indicated (top line) by bars and letters. Residues of the human enzymes that also occur in the quail enzyme are shadowed in the human lines, showing the greatest similarity to be with class I. Dashes indicate gaps. Positional numbers refer to the γ subunit, and therefore also to the horse liver E type model enzyme, but differ from the quail enzyme positional numbers (Figure 2) because of the insertion/deletion of position 118. Residues with functionally important interactions in class I alcohol dehydrogenase are indicated as assigned in Eklund et al. (1987, 1990) from comparisons of the crystallographically analyzed horse liver E-type enzyme and the human enzymes. Bars above the top line indicate positions corresponding to major coenzyme interactions in the horse enzyme (all conserved in quail as shown by thin bars); bars below the top line indicate major substrate interactions (four thick bars for nonconserved residues in quail; thin bars for remaining, conserved residues); letters i, s, and e denote internal, surface or substrate pocket, and external positions, respectively, corresponding to the positions in the subunit interaction area of the class I enzymes. Additional interactions also occur, and all the assignments in the horse enzyme need not apply to the quail enzyme although it is strictly homologous.

the subdivision of the classes (Figure 4), the recognition that the classes evolve at different speeds (Kaiser et al., 1989), and the actual structures (Figure 3) support the notion that the classes in mammals represent separate enzyme functions.

Discussion

Structure Deduced. The structure is based on the recovery of overlapping fragments from all parts of the polypeptide chain (Figure 2). The compositional data (Table I) agree with those from the sum of the sequence analyses. Furthermore, the structure is found to be distantly related to those of other alcohol dehydrogenases and to be compatible with a limited analysis of a few peptides of another avian alcohol dehydrogenase (von Bahr-Lindström et al., 1978). Consequently, the structure deduced appears reliable and clearly establishes the major quail liver alcohol dehydrogenase as a representative of the class I enzyme type but with some features typical of the other classes, class III in particular. The structural properties are consistent with an early subdivision of two lines and a later separation of a third from either of these lines (Figure 4).

Alcohol Dehydrogenase Origin. The present results suggest that the origin of the class distinction may be of the same age as, or older than, the separation of the avian/mammalian line (over 140 million years ago) and suggest the closest and therefore presumably most ancestral relationship to be with the mammalian class I enzymes, in particular the E/γ isozyme type (Figure 3).

The subdivision of the duplications, explaining the presence of the classes (Figure 4), the establishment of class II as the most deviating form, and the interrelationships between class III and either of the other two classes all demonstrate multiple origins and partly mixed properties. The fact that details about the mammalian enzyme origins can be traced from the avian enzyme structure is noteworthy and promises distinctions of still further properties of the mammalian enzyme classes from characterizations of additional submammalian enzyme forms. The mixed properties of the avian enzyme in relation to the mammalian enzyme classes are noticeable at several positions, including those with tryptophan (Figure 3), which influence the UV absorbancy and therefore are directly noticeable in physicochemical properties.

Functional Consequences. Several structural and functional correlations are of special interest. One concerns the overall properties, regarding the conformation at large, the active-site zinc atom, and the loop region around the second zinc atom. The latter region with four Cys zinc ligands (positions 97, 100, 103, and 111) is different in other related proteins such as sorbitol dehydrogenase and ζ-crystallin, which lack the metal,

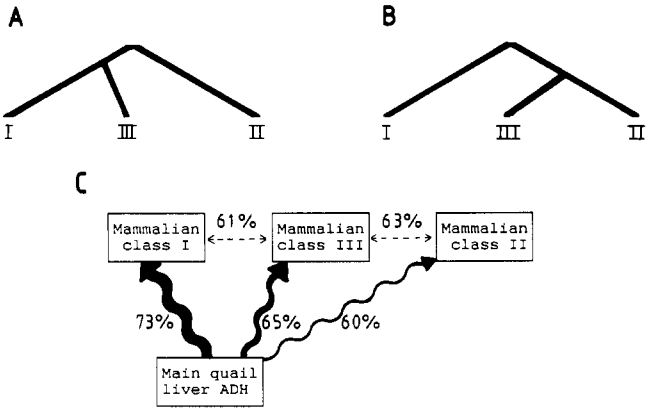

FIGURE 4: Structural relationships suggesting the distinction of two early sublines in the origin of mammalian alcohol dehydrogenase classes. (A, B) Early separation of two lines is shown, followed by a later duplicatory separation of either line into two classes. Alternatives A and B cannot yet be distinguished from the few structures known, but both suggest the presence of two sublevels of duplications in the evolution of the enzyme classes. Initial separation is unlikely to have had classes I and II coevolving because of their wide differences in present-day structures (C). Thus, the main quail liver enzyme now characterized is most closely related to mammalian class I, least related to class II, and intermediately related to class III, as represented by the wavy lines of different widths in C. Numerical values show the extent of residue identities in each case and are from Kaiser et al. (1988) and Figure 3.

the ligands, and for the crystallin even the whole region (Jeffery et al., 1984; Eklund et al., 1985; Borrås et al., 1989). Significantly, this segment with adjacent parts (positions 93–124; Figure 3) is little conserved also in quail liver alcohol dehydrogenase.

Regarding the overall agreements, the avian enzyme shows conservation of all zinc ligands, both those to the active-site zinc atom (Cys-46, His-67, Cys-174) and those to the second zinc atom (Cys-97, -100, -103, -111). Furthermore, there is no drastic amino acid exchange that could largely alter overall properties. Consequently, the conformation of the avian enzyme is likely to be represented by the class I conformational type (Eklund et al., 1987). There are no long insertions and deletions but a one-residue size difference, an insertion before position 119 in relation to the model horse enzyme (Figure 3).

All the residues contributing to the major coenzyme-binding interactions in the class I structure (Eklund et al., 1987) are unchanged in the quail enzyme, as indicated by the thin top bars in Figure 3. Also, in relation to the substrate-binding site (bottom bars, Figure 3), differences are small, but four exchanges occur, Leu (instead of Tyr or Phe) at position 110, Ile (instead of Val or Leu) at position 141, Phe (instead of Leu) at position 309, and Val (instead of Ile) at position 318 (thick bars, Figure 3). Although lack of detectable activity toward methanol and ethylene glycol has been reported for the quail enzyme (Nussrallah et al., 1989), the positional differences do not appear easy to correlate with specific substrate differences, and also for the mammalial class I enzymes, the activities toward methanol and ethylene glycol are fairly low [cf. Eklund et al. (1990)]. The avian enzyme lacks one of the Gly residues previously regarded as strictly conserved (Jörnvall et al., 1987b). Thus, Gly-260 of other characterized alcohol dehydrogenases is replaced in the avian enzyme, showing the corresponding Gly in the dehydrogenase to be dispensable. Gly-260 is also lacking in ζ-crystallin within this protein superfamily (Borrås et al., 1989). However, the largely unaltered substrate-binding properties of the avian enzyme again support the notion that is has a class I type function.

Many differences affect the main subunit–subunit interaction area. In this segment (centered around positions 294–318) no less than 11 (46%) exchanges occur in relation to the class I mammalian alcohol dehydrogenases, and several residues are unique to the quail alternative (Figure 3). This variability is consistent with the fact that the subunit interface is variable also between the mammalian enzyme classes, explaining lack of cross-hybridization (Jörnvall et al., 1987a).

In summary, the avian alcohol dehydrogenase structure differentiates the mammalian enzyme classes into two different sublines and establishes a close connection with the class I type, showing essentially no differences in coenzyme-binding residues and zinc ligands, small substrate-binding differences, and largely unique areas of subunit interactions.

Supplementary Material Available

Table of the total compositions of relevant peptides for the structure deduced (1 page). Ordering information is given on any current masthead page.

Registry No. Alcohol dehydrogenase, 9031-72-5; alcohol dehydrogenase (*Coturnix japonica* major form protein moiety reduced), 127943-50-4.

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Processing of Newly Synthesized Cachectin/Tumor Necrosis Factor in Endotoxin-Stimulated Macrophages†

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ABSTRACT: The biosynthesis and processing of cachetin/tumor necrosis factor (TNF) were examined in the murine macrophage-like cell line RAW 264.7. Lipopolysaccharide-stimulated cells secreted both glycosylated and nonglycosylated 17-kilodalton (kDa) mature cachectin/TNF into the culture medium. Secreted cachectin/TNF was derived from membrane-associated precursors that were precipitated by polyclonal antisera raised against either the mature protein or synthetic peptide fragments of the 79 amino acid cachectin/TNF prohormone sequence. About half of the precursors were N-glycosylated, apparently cotranslationally. The cachectin/TNF precursors were then proteolytically cleaved to release soluble mature cytokine into the medium, while the membrane-bound 14-kDa prosequence remained cell associated. During the period of LPS stimulation, the amount of macrophage cell surface cachectin/TNF remained at a low level, suggesting that both nonglycosylated and glycosylated precursors of cachectin/TNF are efficiently cleaved by these cells. These findings suggest the presence of a unique mechanism for the secretion of cachectin/TNF.

Microbial and parasitic infections and neoplastic diseases in mammals alter the physiological and metabolic state of the host and can advance to cachexia and septic shock (Beisel, 1975). Recently, macrophage-derived polypeptide cytokines (monokines), especially cachectin/tumor necrosis factor (TNF)[1] and interleukin 1 (IL-1), have been implicated as mediators of such metabolic changes in infected hosts [reviewed by Beutler and Cerami (1987) and Dinarello (1984)]. When stimulated by bacterial endotoxin, macrophages secrete large amounts of these cytokines. Cachectin/TNF has been found to mediate catabolic responses in septic animals and to be responsible for endotoxin-induced injury and death (Tracey et al., 1986). Cachectin/TNF has other known in vivo and in vitro effects on tumor cells (Carswell et al., 1975) and participates in host inflammatory responses to viral, bacterial, and parasitic stimuli (Beutler & Cerami, 1987).

Cloning of cDNA for full-length cachectin/TNF mRNA and comparison of its coding sequence with that of the mature, secreted, 17-kDa hormone had revealed that cachectin/TNF is synthesized as a prohormone, whose prosequence is so long that it has not been regarded as a typical "signal" sequence: 76 and 79 amino acid residues for human (Pennica et al., 1984; Shirai et al., 1985) and murine (Fransen et al., 1985; Pennica et al., 1985; Caput et al., 1986) cachectin/TNF's, respectively. The propeptide sequence is highly conserved (86% homologous) between human and mouse proteins, which raised the possibility that it serves a distinct biological function (Beutler & Cerami, 1987). The prosequence has a centrally located hydrophobic region and in this respect resembles other secretory signal sequences (Blobel et al., 1979). However, recent reports indicate unusual features in posttranslational processing of cachectin/TNF as a secretory protein (Muller et al., 1986; Decker et al., 1987; Kriegler et al., 1988). In human monocytes and cells transfected with a cachectin/TNF prohormone cDNA construct, the intact cachectin/TNF prohormone remains associated with the membrane fraction. It was postulated that this long form is clipped to release mature 17-kDa cachectin/TNF into the medium (Muller et al., 1986; Kriegler et al., 1988).

In the present study we investigated the processing of cachectin/TNF in an endotoxin-stimulated murine macrophage-like cell line. Cachectin/TNF was produced initially as a membrane-bound, cell-associated, 26-kDa precursor that was then cleaved to yield soluble, mature, 17-kDa protein, while 14-kDa prosequence peptide remained membrane bound.

Experimental Procedures

Cell Culture and in Vitro Cytotoxicity Assay. RAW 264.7 murine macrophage and L-929 mouse fibroblast lines were obtained from American Type Culture Collection (Rockville, MD). RAW 264.7 cells were grown in RPMI 1640 medium

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[1] Abbreviations: TNF, tumor necrosis factor; IL-1, interleukin 1; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; LPS, lipopolysaccharide; D-PBS, Dulbecco's phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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