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Mammalian dihydroorotase: Nucleotide sequence, peptide sequences, and evolution of the dihydroorotase domain of the multifunctional protein CAD

(aspartate transcarbamylase/expression/pyrimidine biosynthesis/protein domains)

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ABSTRACT **Mammalian DHOase (*S*-dihydroorotate amidohydrolase, EC 3.5.2.3) is part of a large multifunctional protein called CAD, which also has a carbamoyl-phosphate synthetase [carbon-dioxide:L-glutamine amido-ligase (ADP-forming, carbamate-phosphorylating), EC 6.3.5.5] and aspartate transcarbamoylase (carbamoyl-phosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) activities. We sequenced selected restriction fragments of a Syrian hamster CAD cDNA. The deduced amino acid sequence agreed with the sequence of tryptic peptides and the amino acid composition of the DHOase domain isolated by controlled proteolysis of CAD. *Escherichia coli* transformed with a recombinant plasmid containing the cDNA segment 5′ to the aspartate transcarbamoylase coding region expressed a polypeptide recognized by DHOase domain-specific antibodies. Thus, the order of domains within the polypeptide is NH_2–carbamoyl-phosphate synthetase–DHOase–aspartate transcarbamoylase–COOH. The 334-residue DHOase domain has a molecular weight of 36,733 and a pI of 6.1. A fragment of CAD having DHOase activity that was isolated after trypsin digestion has extensions on both the NH_2 (18 residues) and COOH (47–65 residues) termini of this core domain. Three of five conserved histidines are within short, highly conserved regions that may participate in zinc binding. Phylogenetic analysis clustered the monofunctional and fused DHOases separately. Although these families may have arisen by convergent evolution, we favor a model involving DHOase gene duplication and insertion into an ancestral bifunctional locus.**

Dihydroorotase [DHOase; (*S*)-dihydroorotate amidohydrolase, EC 3.5.2.3] catalyzes the synthesis of dihydroorotate from carbamyl aspartate, the third step in mammalian *de novo* pyrimidine biosynthesis. Shoaf and Jones (1) discovered the DHOase from rat ascites cells, copurified as a complex with the first two enzymes of the pathway, glutamine-dependent carbamoyl-phosphate synthetase [CPSase; carbon-dioxide:L-glutamine amido-ligase (ADP-forming, carbamate-phosphorylating), EC 6.3.5.5] and aspartate transcarbamoylase (ATCase; carbamoyl-phosphate:L-aspartate carbamoyl-transferase, EC 2.1.3.2). Coleman *et al.* (2) subsequently found that the three activities were associated with a single 220-kDa polypeptide in Syrian hamster cells. This protein, call CAD or dihydroorotate synthase, is organized into discrete structural domains, each having a distinct function (3–5).

The kinetics, pH dependence, and inhibition of mammalian DHOase have been extensively studied (6–15). Inactivation by cysteine (11) and diethyl thiopyrocarbonate (12) lead to the suggestion that a zinc ion and a histidine side chain, respectively, may participate in catalysis. Christopherson and Jones (12) proposed a catalytic mechanism for DHOase and pointed out several convincing parallels to the zinc proteases that catalyze a formally similar reaction.

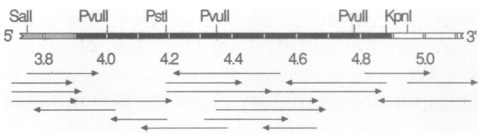

FIG. 1. Nucleotide sequencing strategy. The region of the pCAD142 sequenced is shown schematically: the CPSase domain (stippled bar), the DHOase domain (shaded bar), and the DHOase–ATCase linker (clear bar). Map units represent the distance in kilobases from the start of the cDNA insert. Clones sequenced are indicated by arrows.

We have isolated (16) a 44-kDa fragment from proteolytic digests of CAD that carries only the DHOase activity. The kinetic parameters of the domain and the DHOase activity of CAD are virtually indistinguishable. The isolated DHOase domain, a 88-kDa dimer, has one tightly bound zinc ion per monomer (16), presumably at the active site.

In contrast, the bacterial DHOases are separate monofunctional proteins. First identified in *Clostridium oroticum* (17), the enzyme has been isolated and characterized from this organism (18, 19) and from *Escherichia coli* (20, 21, 23). Both bacterial proteins are zinc-containing dimers. The *Salmonella typhimurium* (22) and *Escherichia coli* (24) DHOase genes and *ura4* (25), which encodes the monofunctional yeast enzyme, have been sequenced, as have the *Drosophila* pyrimidine biosynthetic complex (26) and much of the corresponding *Dictyostelium* gene (27).

Shigesada *et al.* (28) have constructed a cDNA clone, pCAD142, that includes most of the hamster CAD coding region. We have now sequenced† a region of pCAD142 that is shown by complementary protein studies to encode the mammalian DHOase.

METHODS

Subclones of pCAD142 (Fig. 1) restriction fragments were sequenced by the Sanger dideoxy method (29) as described (30). A nested set of subclones was also generated by exonuclease III digestion (31) (Erase-a-Base, Promega).

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Abbreviations: DHOase, dihydroorotase; ATCase, aspartate transcarbamoylase; CPSase, carbamoyl-phosphate synthetase; CAD, a multifunctional polypeptide having CPSase, ATCase, and DHOase activities.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M28866).

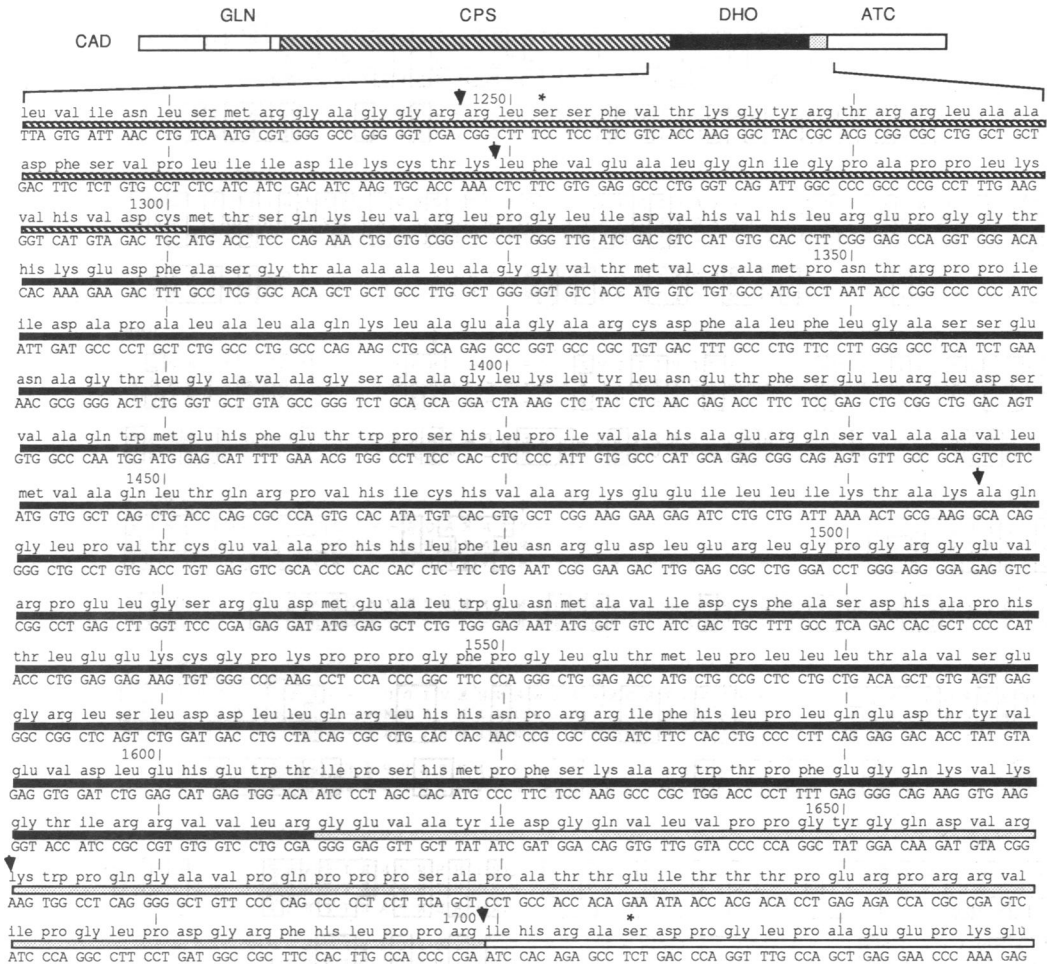

FIG. 2. Nucleotide sequence and deduced amino acid sequence. The core DHOase (DHO) domain, defined on the basis of limited sequence homology, consists of amino acids 1301–1634. The sequence of the COOH terminus of the glutamine-dependent (GLN) CPSase (CPS) domain (hatched bar), the core DHOase domain (solid bar), and part of the DHOase–ATCase (ATC) linker (stippled and clear bar) are shown with the deduced amino acid sequence. The stippled bar indicates the maximum extension on the COOH end of the active DHOase domain isolated from controlled trypsin digests. The phosphorylation sites (34) (*) and the five tryptic cleavage sites (arrows; see Table 3) corresponding to the peptides sequenced are also shown.

Nucleotide and protein sequence analysis was carried out by using BIONET and the IBI programs (IBI). The most parsimonious phylogenetic tree was calculated from the matrix of minimum mutation distances (32). Methods for the isolation of CAD (2), preparation of the DHOase domain from proteolytic digests (16), peptide mapping (16), and immunoblotting (16, 33) have been published.

RESULTS

The nucleotide sequence extending from 3.7 to 5.1 kilobases (kb) of pCAD142 (Fig. 2) has a single open reading frame that also encodes the CPSase (unpublished data) and ATCase (35) domains that flank this region. The hamster amino acid sequence was aligned (Fig. 3) with the monofunctional DHOases from *Saccharomyces cerevisiae*, *E. coli*, and *S. typhimurium* and the sequences of the putative DHOase domain of the *Drosophila* and *Dictyostelium* multifunctional proteins. The sequences of the two prokaryotic enzymes and the yeast enzyme were quite similar (Table 1), with percent identities ranging from 30% to 88%. The hamster, *Drosophila*, and *Dictyostelium* sequences appeared to code for an entirely separate group of homologous proteins (49–55% identities). The alignment (Fig. 3) of these two groups of sequences required numerous, rather extensive insertions and deletions, and, although the overall number of identities was low (14–21%), there were clusters of highly conserved amino acids that helped to establish the register. Surprisingly, much of the long interdomain region connecting the CPSase and ATCase domains of the yeast bifunctional protein (36), which lacks DHOase activity, showed appreciable homology (27–31%) with this region of the hamster, *Drosophila*, and *Dictyostelium* proteins.

The borders of the DHOase domain were defined on the basis of limited sequence homology with the assumption that the mammalian domain is the same size as the monofunctional enzymes. The core domain, so defined (amino acids 1301 through 1634 of Fig. 2), consists of 334 amino acids, has a molecular weight of 36,733, and has a predicted isoelectric point of 6.1.

Comparison of the Putative DHOase Domain with the Proteolytic Fragment. To confirm that this region of pCAD142 encodes DHOase, the DHOase domain was isolated from tryptic digests of CAD and subjected to Edman degradation (H.K., unpublished data). The NH_2-terminal sequence (Table 2) agreed with the deduced sequence starting at residue 1280, 21 amino acids ahead of the core domain. Two peptides isolated from exhaustive trypsin digests (Table 2) were found to map within the region sequenced. The approximate location of the COOH end of the isolated DHOase domain could be determined because peptide 3 maps near one of the CAD phosphorylation sites (residue 1704). Since the proteolytic domain lacked this site (34), the cleavage must occur between residues 1681 (the end of peptide 3) and 1700 (the start of phosphopeptide 2). The calculated molecular mass of the isolated domain ranged between 43.9 and 45.8 kDa—close to the measured value for the elastase and trypsin fragments. The observed amino acid composition, also agreed with the deduced sequence (Table 3). Thus, the active proteolytic fragment isolated from trypsin digests consists of the core DHOase domain with extensions on both the NH_2 (21 residues) and COOH (47–65 residues) ends.

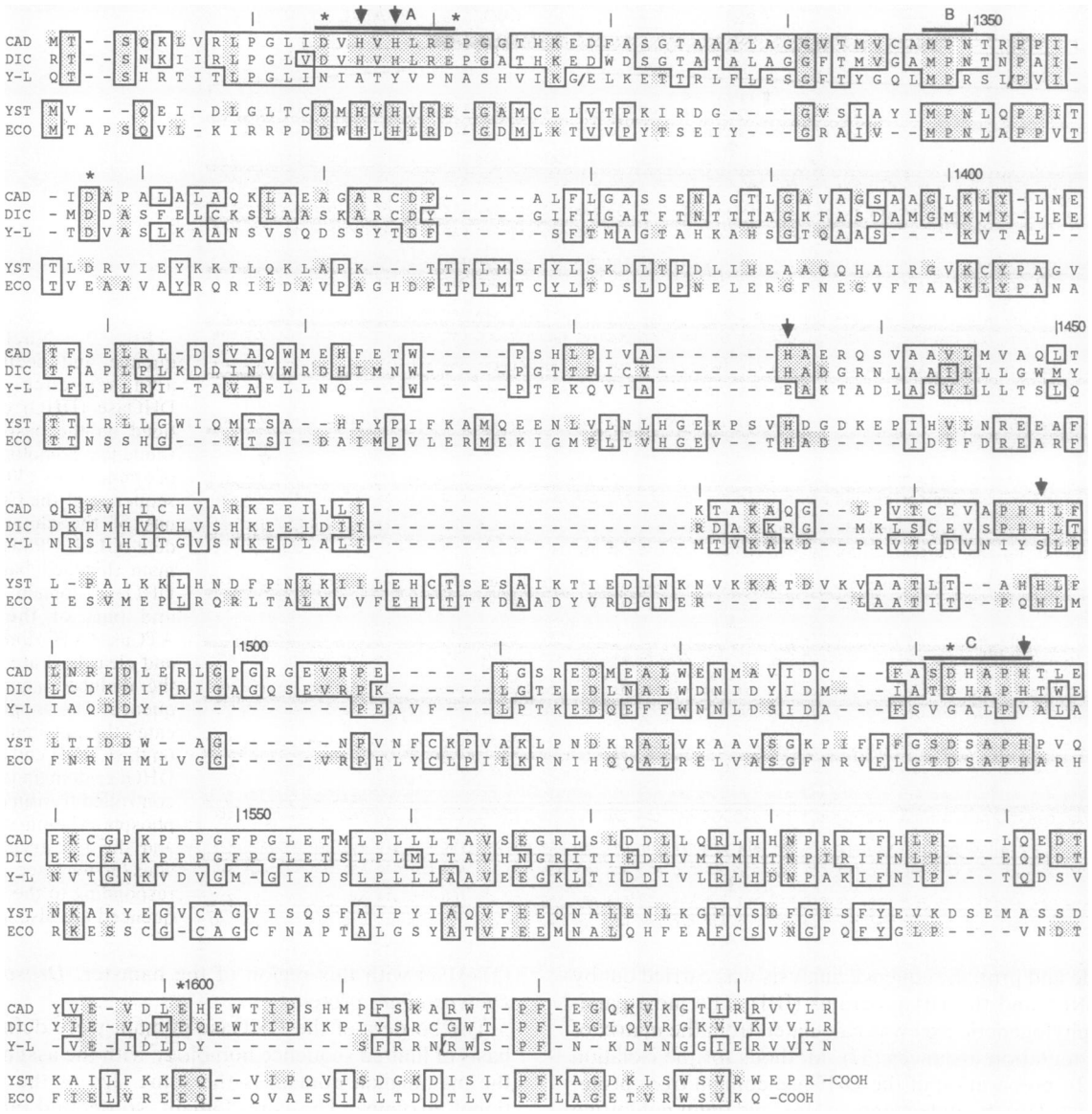

FIG. 3. Alignment of eukaryotic and prokaryotic DHOase sequences. Identical residues in the hamster DHOase (CAD), *D. discoideum* DHOase (DIC) (27), and the yeast interdomain linker (Y-L) (36) are boxed. Identities shared by the *S. cerevisiae* (YST; ref. 25) and *E. coli* (ECO; ref. 24) DHOases are boxed separately. The shaded areas are residues conserved in both groups of sequences. The alignment also included *D. melanogaster* (DRO; ref. 26) and *S. typhimurium* (SAL; ref. 22) DHOases (not shown). Three highly conserved regions, A, B, and C, also noted in the *D. discoideum* sequence (35), are indicated by solid bars. The conserved histidines (arrows) and acidic residues (*) are also shown. Four short segments (/) of the Y-L sequence that have no counterpart in any of the other sequences are not shown.

A 1.8-kb restriction fragment spanning the putative DHOase coding region was subcloned (B.H.Z., unpublished data) into the vector pOTS (37). Immunoblots of extracts from cells transformed with the recombinant plasmid (pBZ28) showed a single species having the expected molecular mass of 43 kDa when probed with antibodies directed against the DHOase domain purified from elastase digests. Cells transformed with the vector gave no bands. Although the expressed protein was found in inclusion bodies and had

Table 1. DHOase sequence identities

	CAD	DRO	DIC	Y-L	YST	ECO	SAL
CAD		185	184	106	67	74	78
DRO	55.4		165	96	59	66	69
DIC	55.1	49.3		93	56	69	71
Y-L	31.4	28.4	27.4		38	34	38
YST	17.2	15.2	14.4	10.0		111	116
ECO	19.5	17.4	18.2	9.1	29.8		307
SAL	20.6	18.2	18.4	10.2	31.1	88.2	

The upper right hand side of the matrix gives the number of identical amino acid residues; the lower half gives the percent identities based on the common length—i.e., (number of identities/length of the shortest sequence of the pair) × 100. References and abbreviations are given in Fig. 3 legend.

Table 2. Peptide sequences in single-letter code

Peptide	Sequence by Edman degradation*	Location	
		Start	End
Amino terminus 1	LFVEALGQIGPAPPL	1280	1295
Peptide 2	AQGLPVTCEVAP	1474	1492
Peptide 3	KWPQGAVPQ	1656	1681
Phosphopeptide 1	RLSSFVTK†	1249	1256
Phosphopeptide 2	IHRASDPGLPAEEPK†	1700	1714

*In some cases only enough of the tryptic peptide was sequenced to allow positive identification; the location numbers refer to the start and end of the entire peptide.

†From Carrey *et al.* (35). All other data are from this report.

Table 3. Calculated (Calc.) and observed (Obs.) amino acid composition of the DHOase domain

Residue	Obs.	Calc.	Residue	Obs.	Calc.
Ala	42	42	Ser	16	16
Val	31	32	Thr	18	23
Leu	47	48	Cys	7	7
Ile	14	15	Tyr	6	4
Pro	—	40	Asx	27	23
Met	4	9	Glx	46	47
Phe	13	12	Lys	22	14
Trp	—	6	Arg	31	25
Gly	35	31	His	18	18

Calculation from the sequence was based on 412 amino acids, residues 1280–1691. Fragments were isolated from elastase digests (16). Some discrepancies are expected because the precise location of the elastase cleavage sites is not known, but the calculated and observed values should still agree within 10%.

no catalytic activity, this result provided good evidence that the clone encodes the mammalian DHOase domain.

Evolutionary Relationships of the DHOases. Despite differences in sequence, the metal content, size, and subunit structure of the mammalian and *E. coli* DHOases are quite similar (Table 4). The evolutionary relationship of the DHOase domains was examined by calculating the most parsimonious phylogenetic tree. The resulting dendrogram clustered the sequences into two distinct groups (Fig. 4). The multifunctional hamster, *Dictyostelium*, and *Drosophila* proteins formed one group, while the monofunctional proteins from yeast, *E. coli*, and *S. typhymurium* constituted a second class of DHOases. The yeast interdomain linker clustered with the multifunctional proteins.

Similarity matrices between five representative zinc proteases and both the hamster and *E. coli* DHOase sequences were calculated. Homology searches of the data banks were also performed by using the three highly conserved regions (Fig. 3) as query sequences. No significant homology could be detected between the DHOases and zinc proteases.

DISCUSSION

Protein sequencing, amino acid analysis, and the expression studies provided direct evidence that the region of pCAD142 extending from 3.9 to 4.9 kb encodes the DHOase domain of CAD. Thus, in mammals the order of the domains from the NH_2 to COOH end of the polypeptide is CPSase–DHOase–ATCase. Our previous model, which tentatively specified an order of DHOase–CPSase–ATCase (16), must therefore be revised.‡ Doubt was cast on the early model by the discovery (38) that a 95-kDa fragment, present in small amounts in elastase digests, reacted with both ATCase and DHOase antibodies, suggesting that these domains are adjacent to one another in the CAD polypeptide (38).

Sequence homology clearly established the location of the CPSase and ATCase domains in the *Drosophila* gene (26), but the assignment of the DHOase coding sequence was not as convincing because of the limited similarity to the *E. coli* gene. Moreover *Drosophila* probes from the postulated DHOase domain did not hybridize with hamster CAD genomic clones or with pCAD142. This interpretation also disagreed with genetic and biochemical analyses (39, 40), which placed the DHOase domain at the distal (5′) end of the *Drosophila* locus. Although DHOase copurifed with the

‡We had placed the DHOase domain at the amino terminus of the polypeptide because repeated Edman degradation attempts indicated that the isolated domain, like the parent protein, had a blocked amino terminus—a result we now believe to have been a sequencing artifact.

Table 4. Properties of CAD and *E. coli* DHOase

Property of DHOase	Hamster*	*E. coli*†
Molecular weight	44,000 (36,733)	38,400 (38,824)
Amino acid residues	334	348
Isoelectric point	5.1 (6.1)	5.0, 5.3 (5.7)
Zn (eq/mol of monomer)	1.0	0.95
Subunit structure	monomer/dimer	monomer/dimer
Dimer M_r	88,000	80,900

*The measured properties are for the CAD DHOase domain isolated from elastase digests (16); calculated values, in parenthesis, are based on the core domain defined in this study.
†Properties of *E. coli* DHOase (21); values in parentheses are calculated from the sequence (24).

CPSase and ATCase activities in *Drosophila* (41), a complex of two different polypeptides could not be ruled out. Similarly, *Dictyostelium* DHOase has not been isolated, and the assignment (27) was based on strong homology, to the *Drosophila* sequence.

The *ura* genes illustrate that identification of a functional gene based solely on sequence homology could be misleading. The interdomain linker of the yeast bifunctional protein, which lacks DHOase activity, is clearly homologous to the hamster, *Drosophila*, and *Dictyostelium* DHOases, while the sequence of the *ura4* gene that codes for the active yeast enzyme is appreciably different. The agreement of the nucleotide sequencing and protein studies reported here clearly establishes the identity of the CAD DHOase domain and supports the *Drosophila* and *Dictyostelium* assignments.

The DHOases lacked the zinc signature sequence found in many of zinc proteases (42), and no other obvious structural relationships were detected. Common catalytic zinc ligands include histidine, glutamate (or aspartate), and cysteine (43). None of the cysteines in CAD DHOase were conserved, but 5 of the 18 histidines (Fig. 3) are found in all of the DHOases sequenced. Three histidines and three of five invariant acidic residues occur within two short, highly conserved sequences (Fig. 3, regions A and C). Although a data base search for homologs containing region A and C sequences failed to

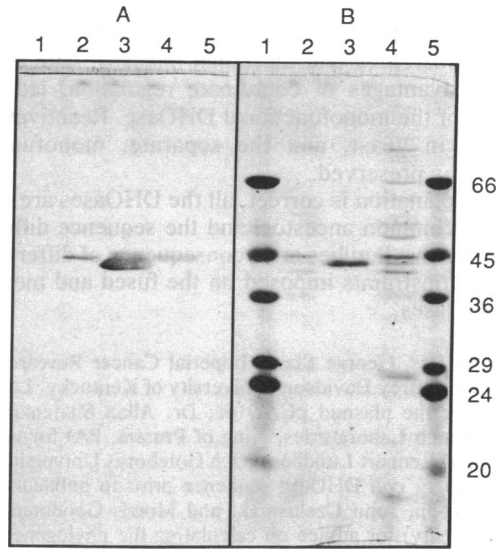

FIG. 4. Immunoblot of the tryptic and recombinant DHOase domains. SDS/PAGE gels of the isolated DHOase domain (lanes 3) and extracts from *E. coli* cells transformed with the recombinant plasmid pBZ28 (lanes 2 and 4) were electroblotted onto nitrocellulose. The blot (*A*) was probed with CAD DHOase domain antibodies (32) and then stained (*B*) with amido black. The molecular masses in kDa of standard proteins (lanes 1 and 5) are also shown.

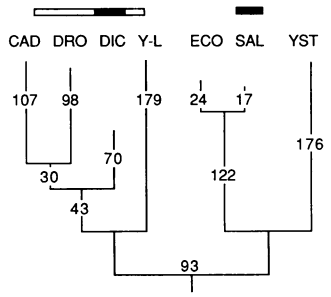

FIG. 5. Dendogram of the DHOase domain. The evolutionary relationships (see Fig. 3 for references and abbreviations) were determined by the method of maximum parsimony (31). The bar diagrams identify the proteins as either monofunctional.

retrieve any known metalloproteins, these regions are prime candidates for the catalytic zinc-binding sites.

Faure *et al.* (27) noted that the DHOases fall into two distinct families. The phylogenetic analysis (Fig. 5) clearly shows that the monofunctional and fused DHOases have a different evolutionary history, since the dendrogram does not conform to the accepted phylogeny of the organisms represented. Although the *Dictyostelium* lineage predates the major radiation that leads to fungi, plants, and animals (44), the dendrogram clusters the enzyme from *Dictyostelium* with higher eukaryotes and the yeast enzyme with the prokaryotic DHOases. As discussed by Freund and Jarry (26) and Faure *et al.* (27), the fused DHOases may have evolved separately, perhaps as descendants of a long spacer region separating the CPSase and ATCase domains in a common ancestral gene resembling the yeast *ura2* locus. The two DHOase families, which share only 20% sequence identity, are certainly different enough to suggest convergent evolution.

However, divergent evolution provides an equally plausible explanation. According to this model, the fusion of CPSase and ATCase genes, separated by an ≈300-base-pair spacer, occurred sometime between the divergence of bacteria and the slime molds. This early event was followed by duplication of a monofunctional DHOase gene, one copy of which was translocated and inserted into the spacer region. Perhaps initially nonfunctional reactivation of the fused gene in the *Dictyostelium* and metazoan lineages, with the concomitant advantages of coordinate regulation, led to the extinction of the monofunctional DHOase. Reactivation did not occur in yeast, and the separate, monofunctional DHOase was preserved.

If this explanation is correct, all the DHOases are descendants of a common ancestor, and the sequence differences between the two families are a consequence of differences in structural constraints imposed on the fused and monofunctional DHOases.

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