Cloning, Overexpression, and Characterization of the Functional Dihydroorotase Domain of the Mammalian Multifunctional Protein CAD†

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ABSTRACT: Mammalian dihydroorotase (DHOase) is part of a large multidomain protein called CAD, which initiates the first three steps in the de novo pyrimidine biosynthetic pathway. DHOase activity is carried out by a 44-kDa structural domain which could be isolated in active form from elastase digests. A core domain the same size as monofunctional dihydroorotases was defined, although the domain borders were uncertain. Two recombinants were overexpressed in *Escherichia coli*. The first encoded the core domain with 55 and 13 residues added to the amino and carboxyl ends, respectively, and was expressed in insoluble form. The recombinant protein was refolded from urea into a soluble form which was resistant to protease digestion but was catalytically inactive. In contrast, the proteolytic fragment from CAD could be unfolded and refolded with recovery of 40–100% catalytic activity. The second construct, which approximated the proteolytic fragment, had 21 residues on the amino end and 65 residues on the carboxyl end of the core domain. A 46-kDa soluble protein was expressed at 4% of the total soluble protein. The recombinant protein was catalytically active and had the expected amino-terminal sequence. The protein was purified to homogeneity. Dihydroorotase saturation curves gave a K_m = 41.9 ± 3.5 μM and a k_{cat} = 2.79 ± 0.06 s^{-1}, parameters that were similar to those obtained for the proteolytic fragment. The K_m was 6-fold higher and the k_{cat} 2-fold lower than the values obtained for the parent protein, which suggests that interdomain interactions stabilize the active conformation. These studies more precisely define the functional DHOase domain and provide a clone suitable for mutagenesis and mechanistic studies.

Dihydroorotase (DHOase, EC 3.5.2.3)[1] catalyzes the reversible interconversion of carbamyl aspartate and dihydroorotate in the third step of the mammalian de novo pyrimidine biosynthetic pathway (Jones, 1980). In bacteria (Sander & Heeb, 1971; Washabaugh & Collins, 1986) and yeast (Sander et al., 1965; Taylor et al., 1976), the enzyme is monofunctional, but in higher eukaryotes, the dihydroorotase activity is associated with a large multifunctional protein called CAD (Shoaf & Jones, 1973; Mori et al., 1975; Coleman et al., 1977). The enzymes which catalyze the first two reactions of the pyrimidine biosynthetic pathway are also associated with CAD: glutamine-dependent carbamyl-phosphate synthetase (CPSase, EC 6.3.5.5) and aspartate transcarbamylase (ATCase, EC 2.1.3.2). On the basis of kinetic, inhibition, and chemical modification studies, Christopherson and Jones (1979, 1980) proposed that a zinc ion and a histidine residue are involved in DHOase activity, and have suggested a plausible catalytic mechanism.

A 44-kDa proteolytic fragment (Kelly et al., 1986) of the CAD polypeptide which could catalyze the dihydroorotase reaction, but which had none of the other catalytic activities, was isolated from controlled elastase digests. The fully functional fragment contains a tightly bound zinc ion essential for catalysis, and exists in a concentration-dependent equilibrium between monomer and dimer. Thus, the dihydroorotate activity is associated with a stable, autonomous domain of the CAD polypeptide.

We have sequenced two overlapping cDNA clones encoding the entire CAD polypeptide (Simmer et al., 1989, 1990a,b; Bein et al., 1991). The region encoding the DHOase domain was identified by mapping the location of peptides, isolated from exhaustive trypsin digest of the DHOase proteolytic fragment, in the cDNA sequence (Kelly et al., 1986; Simmer et al., 1990b). Subclones containing this region of the CAD coding sequence expressed a protein which reacts with antibodies directed against the isolated DHOase domain (Simmer et al., 1990b).

While most of the functional domains of CAD have sequences which are clearly homologous to their bacterial counterparts, the DHOase domain is an exception. The mammalian and *Escherichia coli* dihydroorotases share only 20% sequence identity, although there are clusters of residues suspected to be involved in catalysis which allowed alignment of their sequences (Simmer et al., 1990b). There appear to be two distinct classes of dihydroorotase (Simmer et al., 1990b). Monofunctional proteins from *E. coli* (Backstrom et al., 1986), *Salmonella typhmurium* (Neuhard et al., 1986), and yeast (Guyonvarch et al., 1988) constitute one class of homologous proteins, while the dihydroorotase domains of multifunctional proteins from mammals (Simmer et al., 1990b), drosophila (Freund & Jarry, 1987), and dictyostelium (Faure et al., 1989) form a separate class. The lack of sequence homology with a monofunctional protein makes the assignment of the precise dihydroorotase domain borders of the multifunctional proteins uncertain. A 38-kDa core domain, the same size as the *E. coli* enzyme (Simmer et al., 1990b), was tentatively defined in CAD.

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[1] Abbreviations: CAD, multifunctional protein that catalyzes the first three steps in de novo pyrimidine biosynthesis in higher eukaryotic organisms; ATCase, aspartate transcarbamylase (EC 2.1.3.2); CPSase, carbamyl-phosphate synthetase (EC 6.3.5.5); DHOase, dihydroorotase (EC 3.5.2.3); DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; kDa, kilodalton(s); HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris-(hydroxymethyl)aminomethane.

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Musmanno and co-workers (Musmanno et al., 1991) constructed a DHOase–ATCase clone which could complement *pyr*C-deficient *E. coli* mutants and had detectable activity in cell extracts. By constructing a series of deletion mutants, they showed that the maximum activity required a portion of the interdomain linker. Williams and co-workers (Williams et al., 1990) expressed a cDNA fragment encoding the CAD DHOase domain which was inactive.

We report here the cloning, overexpression, and purification of two recombinants encoding only the DHOase domain of CAD, which differ in size and sequence at the amino and carboxyl ends. Only one of the two recombinant domains, pBZ22, could fold into a catalytically active species. Although the smaller recombinant, pBZ28, is expressed at high levels and can be refolded into a stable, soluble protein, it lacks catalytic activity. The active recombinant has kinetic parameters that are similar to those of the isolated parent domain.

EXPERIMENTAL PROCEDURES

Materials. Klenow fragment, mung bean nuclease, restriction enzymes, agarose, and acrylamide were obtained from Bethesda Research Laboratories. Calf intestinal phosphatase was purchased from Boehringer Mannheim, trypsin TPCK was from Worthington, *Bam*HI linkers were from Stratagene, and casamino acids were from Difco. Bradford protein assay reagent and Ag 501-X8 mixed-bed resin were purchased from Bio-Rad; Immobilon-P and nitrocellulose were from Millipore and Schleicher & Schuell, respectively, while electrophoresis molecular weight standards were purchased from Sigma Chemical Co. PCR oligonucleotides were synthesized by the Wayne State Macromolecular Core Facility.

CAD DHOase domain was prepared according to Kelly and co-workers (Kelly et al., 1986), and *E. coli* DHOase was purified by the method of Washabaugh and Collins (1984).

Expression Vectors and E. coli Strains. pOTSΔF33 and *E. coli* strains AR120 and AR68 were gifts from Dr. A. R. Shatzman (Smith Kline & French Laboratories) (Shatzman & Rosenberg, 1987). The pOTSΔF33-derived clones in AR120 and AR68 were grown under a variety of conditions and induced with nalidixic acid on maximal induction medium (Mott et al, 1985).

Heat shock inductions of pJLA503 (Schauder et al., 1987) derived clones in *E. coli* strains HB101 and AR68 were performed by adding an equal volume of media at 63 °C to cells growing at 28 °C.

Both pEK81 and *E. coli* strain EK1104 were gifts from Dr. E. R. Kantrowitz (Nowlan & Kantrowitz, 1985) with the pEK81 being derived from pEK2 (Nowlan & Kantrowitz, 1985). The EK1104 cells were grown on minimal media (Dr. E. R. Kantrowitz, personal communication) composed of an autoclaved solution containing 42.2 mM Na_2HPO_4, 22 mM KH_2PO_4, 19 mM NH_4Cl, 9 mM NaCl, and 2.5% casamino acids supplemented with filter-sterilized stock solutions to give the following final concentrations: 0.4% glucose, 0.5 μg/mL $ZnSO_4 \cdot 7H_2O$, 0.1 mM $CaCl_2$, 0.5 μg/mL $FeSO_4 \cdot 7H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 5 μg/mL thiamin, 0.001% tryptophan, and 8–12 μg/mL uracil. When the host cells contained plasmids, 100 μg/mL ampicillin was added to the media.

The dihydroorotase-deficient *E. coli* strain X7014, cgsc 5358 (Semple & Siebert, 1975), was obtained from the Yale *E. coli* Genetic Stock Center. Cultures were grown on 2×YT media or on EK1104 minimal media supplemented with 50 μg/mL adenine, with 200 μg/mL streptomycin, with thiamin increased to 25 μg/mL, and with glucose replaced by 0.4% glycerol. When the host cells contained plasmids, 100 μg/mL ampicillin was added to the media.

A clone carrying the monofunctional *E. coli* dihydroorotase, pKC16*pyr*C, was a gift from Dr. K. D. Collins (University of Maryland) (Washabaugh & Collins, 1986).

Construction of Plasmids. Cloning techniques described in standard texts were used to construct the plasmids shown in Figure 1. Transformation of bacterial cells was carried out by the standard transformation protocol of Hanahan (1985). During transformation of cells with pJLA503-derived vectors, the heat shock step was changed to 35 °C for 7 min.

The DHOase coding insert of clone pBZ13 was synthesized by PCR in a Perkin Elmer thermal cycler using two oligonucleotides flanking the DHOase domain: 5′ACTTATTGGC*ATG*CTCTTCGTGGAGGCCCTGGGTC3′ (N-terminal) and 5′GTTTGTCGAC*TCA*TCGGGGTGGCAAGTGGAAGCGG3′ (C-terminal), where the CAD DHOase sequence is shown in boldface type, added restriction sites *Sph*I (N-terminal) and *Sal*I (C-terminal) are underlined, and start and stop codons are italicized. PCR products from several different tubes were combined for use in cloning.

Harvesting Cells and Sonication. Cell cultures were cooled on ice and centrifuged at 25000*g*. Cell pellets were washed in 20 mM HEPES, pH 7.4, and then resuspended at 1 g wet weight cells/3.3 mL of sonication buffer (50 mM Tris–acetate, pH 8.3, 10% glycerol, 50 mM NaCl, 50 μM $ZnCl_2$, and 1 mM DTT). PMSF was added just prior to sonication to a final concentration of 1 mM using a stock solution of 150 mM PMSF in 2-propanol. Cells were sonicated with a Branson Model W-350 sonifier fitted with a microtip and set at 50% duty cycle, pulsed mode, output control 6. Solutions of 10 mL or more were sonicated 5 × 3 min, while volumes of 1.0 mL were sonicated 6 × 20 s. During sonication, solutions were kept on ice to eliminate heating.

Refolding Procedure for the pBZ28 Recombinant Protein. *E. coli* AR120 cells transformed with pBZ28 were harvested 20–25-h postinduction. Cells were pelleted, washed, and sonicated as described above. The sonicate was diluted with 10 volumes of wash buffer (20 mM HEPES, pH 7.0) and centrifuged at 5500*g* for 15 min. Pellets were washed twice with wash buffer and were resuspended in solubilization buffer (7 M urea, 50 mM Tris–acetate, pH 8, and 50 mM 2-mercaptoethanol) at 1.25 mL/g wet weight. The 10 M urea stock solution used to prepare solubilization buffer was made fresh and preincubated with AG 501-X8 resin before use. After incubation for 30 min at room temperature, the solution was centrifuged at 16000*g*, 25 °C, to remove undissolved material and diluted with 3 volumes of dilution buffer (50 mM Tris–acetate, pH 8, 50 mM 2-mercaptoethanol, and 50 μM $ZnCl_2$). The diluted urea extract was dialyzed in the cold room, first against 50 mM Tris-HCl, pH 8, 1 mM 2-mercaptoethanol, 5% glycerol, 50 μM $ZnCl_2$, and 0.1 M urea and then against three changes of 50 mM Tris-HCl, pH 7.4, 1 mM 2-mercaptoethanol, 5% glycerol, and 50 μM $ZnCl_2$. Solutions of solubilized protein were centrifuged for 20 min at 100000*g* in a Beckman L5-65 ultracentrifuge to remove insoluble material.

Purification of the pBZ22 Recombinant Protein. *E. coli* EK1104 cells transformed with pBZ22 were harvested 25 h after inoculation. Cells from 800 mL of culture were pelleted, washed, resuspended in 25 mL of sonication buffer, and sonicated as described above. The sonicate was then centrifuged for 10 min at 16000*g* at 4 °C. Ammonium sulfate was added to the sonicate supernatant to 45% saturation, and, after 30 min on ice, the solution was centrifuged for 25 min

at 14 000 rpm. The 45% ammonium sulfate pellet was then resuspended in 18 mL of 50 mM HEPES, pH 7.4, 5% glycerol, and 0.5 mM DTT and centrifuged for 5 min at 16000*g* at 4 °C. The supernatant was dialyzed at 4 °C against 1 L of 50 mM HEPES, pH 7.4, 5% glycerol, and 0.5 mM DTT, with two changes, and then against 1 L of 35 mM HEPES, pH 7.4, 5% glycerol, and 0.5 mM DTT with two changes. The dialyzed solution was loaded onto a 10-mL DEAE-Sephacel column which had been equilibrated with 50 mM HEPES, pH 7.4, 1 mM DTT, and 5% glycerol. After the column was washed with 30 mL of column buffer, the solubilized protein was eluted with 70 mL of a 0–0.28 M NaCl gradient.

Tryptic Digests of the CAD DHOase Proteolytic Fragment and Solubilized pBZ28 Recombinant Protein. The CAD DHOase proteolytic fragment and solubilized pBZ28 recombinant protein were incubated with trypsin (50 μg of protein/1 μg of trypsin) from 0 to 120 min in 50 mM Tris-HCl, pH 7.4, 1 mM DTT, 5% glycerol, and 150 mM NaCl. Aliquots were withdrawn at intervals and quenched by the addition of SDS–PAGE sample buffer. Trypsin TPCK was quantitated in 50 mM ammonium bicarbonate, pH 8.5, using $\epsilon^{1\%,280} = 14.3$ (Worthington).

Electrophoresis and Electroblotting. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out on 1.5-mm, 7.5–15% gradient slab gels with 5% stacking gels using the buffer system described by Laemmli (1970). Gels were run using a Hoeffer Model SE650 gel apparatus and stained with Coomassie Brilliant Blue R.

Protein was transferred from SDS–polyacrylamide gels to an Immobilon-P (Millipore) membrane or to nitrocellulose using a homemade electrotransfer unit for a minimum of 3 h at 6 V/cm.

Immobilon-P membranes were prepared for transfer according to the manufacturer's instructions. Membranes were stained with 0.1% Coomassie Brilliant Blue R (w/v)/50% methanol and destained with 45% methanol. HPLC-grade methanol was used for all solutions. Bands containing a total of ~100 pmol of protein were excised from the membrane and submitted to the Wayne State Macromolecular Core facility for sequencing.

After electrotransfer, nitrocellulose membranes were incubated 15 min with 3% nonfat dry milk in TBS (Tris-buffered saline: 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl), washed 10 min in TBS/Tween (TBS containing 0.05% Tween 20), and incubated for 1 h at 37 °C in TBS/Tween with the appropriate primary antibody at a dilution of 1/26 000. After incubation, the nitrocullulose was washed 4 times with TBS/Tween, incubated at room temperature with anti-rabbit peroxidase conjugate (Sigma) at a dilution of 1/1000, washed twice with TBS/Tween, and washed twice with TBS. Antibody binding bands were then visualized by incubating the nitrocellulose in a TBS solution containing 0.012% H_2O_2, 0.6 mg/mL 4-chloro-1-naphthol, and 20% methanol. In some cases, the nitrocellulose was stained with 0.1% Amido black, 50% methanol, and 10% acetic acid and destained with 50% methanol/10% acetic acid.

Protein Quantitation. Protein was measured by the method of Bradford (1976) with reagents from Bio-Rad, using bovine serum albumin as a standard.

Enzyme Activity and Kinetics. Dihydroorotase activity was assayed spectrophotometrically in the degradative direction by measuring carbamyl aspartate with the colorimetric assay of Prescott and Jones (1969) or by measuring the rate of disappearance of dihydro-L-orotate, using an extinction coefficient $\epsilon_{230nm} = 1.17\ mM^{-1}\ cm^{-1}$ (Washabaugh & Collins, 1984). Spectroscopic measurements were made on a Perkin Elmer Model lambda 5 UV/Vis spectrophotometer equipped with thermostatted cuvette holders.

Data for the dihydroorotate saturation curve of the pBZ22 recombinant protein were obtained using the colorimetric assay (Prescott & Jones, 1969). Assay mixtures containing 1 mL of 100 mM Tris–acetate, pH 8.3, and varying concentrations of dihydro-L-orotate were preincubated at 37 °C for 10 min. Reactions were initiated by adding 6.5 μL of purified protein (0.73 μg), incubated for 20 min, and then quenched. Kinetic parameters were obtained by nonlinear least-squares fit to the Michaelis–Menten equation using the program MINSQ (Micromath).

Amino Acid Sequencing. Amino acid microsequence analysis was obtained by automated Edman chemistry on an Applied Biosystems gas-phase sequenator (Model 470) with on-line HPLC (Model 120) and a Nelson analytical chromatography data system in the Wayne State Macromolecular Core Facility.

Preparation of Polyclonal Antibodies. E. coli DHOase antibodies were prepared as described previously (Grayson et al., 1985) by immunizing a New Zealand white rabbit with purified *E. coli* DHOase.

RESULTS

Clone pBZ28. We recently defined a segment of CAD cDNA as the DHOase core domain on the basis of its homology with published sequences of other dihydroorotases (Simmer et al., 1990b). pBZ28 was constructed using the closest available restriction sites flanking this core domain: *Hin*cII (5′ site, nucleotide 4208)[2] and *Cla*I (3′ site, nucleotide 5386). The *Hin*cII/*Cla*I fragment was blunt-end-ligated into pOTSΔF33, an expression vector under the control of the λ promoters with start and stop codons in all three reading frames (Figure 1). *E. coli* AR120 cells transformed with pBZ28 expressed a new protein of the expected molecular mass (44.2 kDa) as early as 2 h after nalidixic acid induction (Figure 2). The recombinant protein was catalytically inactive, and low-speed centrifugation (500*g*) of cell sonicates showed that it was insoluble. Short induction times (2–5 h), low growth temperatures (25–35 °C), and addition of zinc to the growth medium (100 μM) did not affect the recombinant's solubility. Immunoblots showed a band of ~44 kDa which reacted with antibodies directed against the tryptic CAD DHOase domain (see Figure 3; Simmer et al., 1990b). The band was not observed in induced cells transformed with the parent vector. Amino acid sequencing of the first eight amino acids of the recombinant protein gave the predicted N-terminal sequence, MDRRLSSF, where the underlined amino acids are contributed by the vector (Figure 1). Some preparations of the recombinant exhibited two bands on SDS gels. Sequencing of the faster migrating band showed that it started 16 amino acids downstream of the predicted N-terminal and suggests that proteolysis occurs under some conditions.

The recombinant protein was produced in large amounts and could be isolated in a relatively pure form by low-speed

[2] The region corresponding to the dihydroorotase domain of hamster CAD was sequenced by Simmer and co-workers (Simmer et al., 1990b) using pCAD142 (Shigesada et al., 1985), a cDNA clone containing most of the hamster CAD coding region. pCAD142 is missing approximately 0.5 kb of the CAD coding sequence corresponding to the 5′ end of the mRNA. The missing region has recently been sequenced (Bein et al., 1991), and the numbering of the CAD sequence was changed to incorporate the additional sequence. In this paper, the numbering of Bein and co-workers (Bein et al., 1991) is used. To convert the amino acid numbering used by Simmer and co-workers (Simmer et al., 1990b) to the new numbering system, add 155 residues.

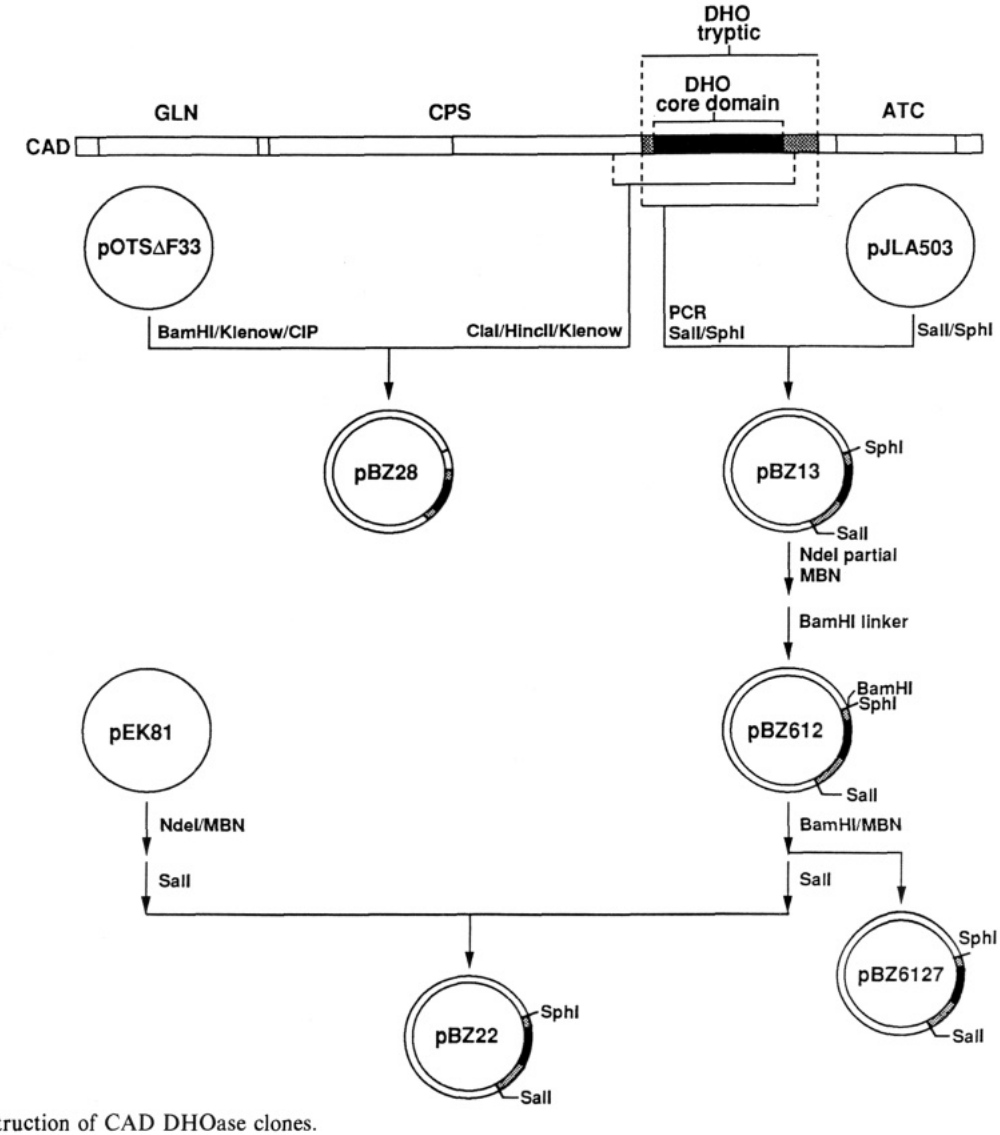

FIGURE 1: Construction of CAD DHOase clones.

centrifugation. The protein was solubilized and refolded as described under Experimental Procedures.

Although the soluble refolded protein was inactive, it showed a resistance to digestion by trypsin (data not shown) similar to that exhibited by the CAD proteolytic DHOase (Kim et al., 1992). Refolding in the presence of stoichiometric amounts of the purified CAD ATCase domain, or in the presence of a mixture of CAD N-terminal tryptic fragments (80, 50, and 30 kDa) (Kim et al., 1992), did not yield active protein. Attempts to refold into an active form by adding zinc (50 μM $ZnCl_2$) or substrate (2.5 mM carbamyl aspartate, 156 μM dihydroorotate) were unsuccessful. Refolding under conditions which stabilize the *E. coli* monofunctional dihydroorotase (refolding under N_2 and in the absence of reductants) (Washabaugh & Collins, 1984) or which stabilize intact CAD (30% dimethyl sulfoxide) did not yield active protein.

Refolding experiments were performed on the purified CAD proteolytic DHOase domain under conditions essentially the same as those used for recombinant protein refolding, and resulted in the recovery of ~20% of the total protein after the dialysis. The activity of the refolded domain ranged between 40 and 100% of the specific activity of the untreated domain.

The pBZ28 recombinant protein differs from the tryptic DHOase in that it carries an extra 34 amino acids on the N-terminal (including 3 from the vector), is shorter by 46 amino acids on the C-terminal, and terminantes in 8 amino acids contributed by the vector. Since the missing and added sequences of pBZ28 may have caused incorrect folding, and, in turn, an inactive protein, we decided to construct a clone which corresponded to the sequence of the DHOase domain produced by trypsin digestion of CAD.

Clones pBZ13 and pBZ6127. An insert corresponding to the tryptic CAD DHOase (nucleotides 4303–5562) was synthesized by PCR using pCAD142 as a template. The N-terminal primer contained an *Sph*I restriction site and a start codon upstream of the CAD DHOase sequence, while the C-terminal primer provided a stop codon following by a *Sal*I restriction site. The insert was ligated into pJLA503 (Schauder et al., 1987), an expression vector under the control of the λ promoters which carries the λ cIts857 gene (Figure 1). Heat shock induction of the resulting recombinant, pBZ13, produced a small amount of a soluble ~46-kDa protein which reacted with antibodies directed against CAD DHOase (Figure 3). The levels of recombinant expressed did not increase when AR68, a protease-deficient *E. coli* strain, was used as the host.

To construct recombinant pBZ6127, an ATG upstream of the start codon in pBZ13 was eliminated, while retaining the same distance between the start codon and the ribosome binding site (Figure 1). Cells transformed with pBZ6127 exhibited expression levels similar to cells transformed with pBZ13. In both cases, activities could not be distinguished

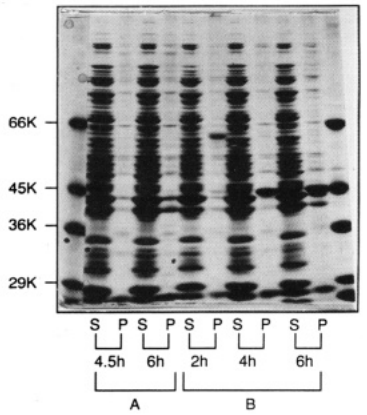

FIGURE 2: SDS–polyacrylamide gel of cell sonicates from cultures transformed with pOTSΔF33 or with pBZ28. *E. coli* AR120 cells transformed with the parent vector pOTSΔF33 (panel A) or with pBZ28 (panel B) were harvested 2, 4, 4.5, and 6 h after nalidixic acid induction. Cells were sonicated and then centrifuged at 12000*g* to obtain supernatants (S) and pellets (P). A new band, of the size expected for the recombinant protein (44.2K), appears in the pellets of pBZ28-transformed cells as early as 2 h after induction, and is not present in the control. The band appearing at ~64 kDa in the 2-h pellet of pBZ28 does not react with polyclonal antibodies directed against the CAD proteolytic DHOase domain. Dihydroorotase activities of the parent vector and clone-transformed cells were not significantly different. Reduced growth temperature (25 °C) and addition of zinc to the growth media did not result in the expression of the protein in active or soluble form.

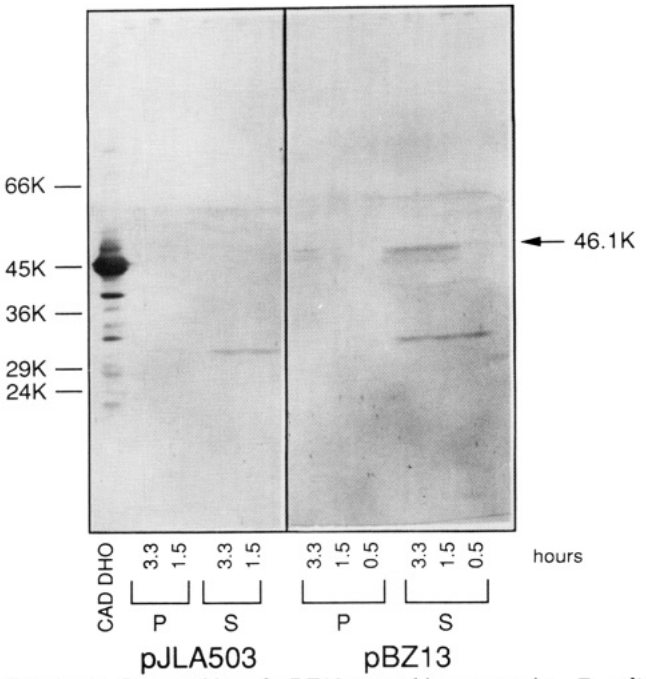

FIGURE 3: Immunoblot of pBZ13 recombinant protein. *E. coli* HB101 cells transformed with parent vector pJLA503 or with clone pBZ13 were harvested 0.5, 1.5, and 3.3 h after heat induction. Cells were sonicated and then centrifuged at 12000*g* to obtain supernatants (S) and pellets (P). SDS–polyacrylamide gels of the centrifuged sonicates and the CAD DHOase domain produced by elastase were electroblotted onto nitrocellulose. The blot was exposed to polyclonal antibodies directed against the CAD proteolytic DHOase domain. A band of the expected size (arrow), which is not present in sonicates of the parent vector, reacts with the antibodies.

from background levels. Moreover, clone pBZ13 did not effectively complement the dihydroorotate deficiency of *E. coli* strain X7014.

Clone pBZ22. In order to increase expression of the recombinant protein, we turned to a different expression system. pEK81 is a plasmid carrying the *E. coli pyr*BI genes

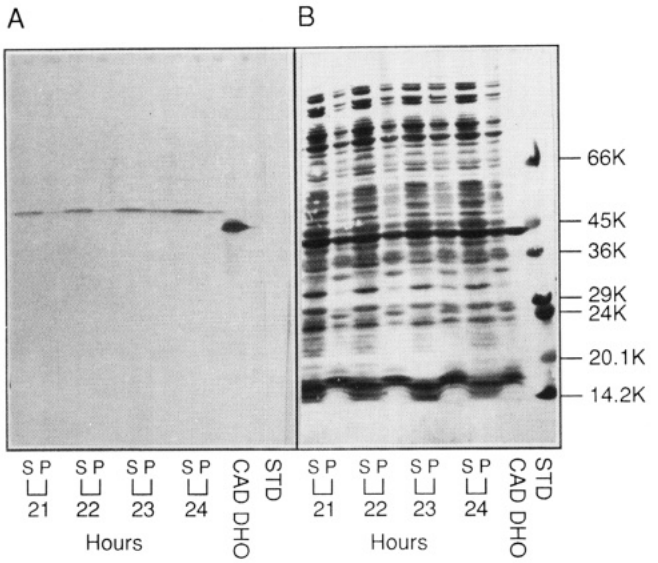

FIGURE 4: Immunoblot of pBZ22 recombinant protein harvested 21–24 h after inoculation. *E. coli* EK1104 cells transformed with pBZ22 were harvested 21, 22, 23, and 24 h after inoculation. Cells were sonicated and then centrifuged at 16000*g* to obtain supernatants (S) and pellets (P). SDS–polyacrylamide gels of the centrifuged sonicates and the CAD DHOase domain were electroblotted onto nitrocellulose. The blot was exposed to polyclonal antibodies directed against the tryptic dihydroorotase domain (panel A) and then stained with amido black (panel B). A band of the expected size (arrow) reacts with the antibodies.

which encode the catalytic and regulatory polypeptides of *E. coli* aspartate transcarbamylase, inserted downstream of the *pyr*BI regulatory region (Nowlan & Kantrowitz, 1985). Expression is induced by growing transformed host cells on minimal media with limited amounts of uracil. Upon depletion of uracil, the *pyr*BI gene products are expressed.

The DHOase insert was cut out of pBZ6127 with *Bam*HI and *Sal*I and ligated into pEK81 from which the *pyr*BI gene had been excised (Figure 1). The resulting construct, pBZ22, was transformed into EK1104 cells, grown on minimal media containing 12 μg of uracil/mL, and harvested 18–30 h after inoculation. An immunoblot of supernatants and pellets of cell sonicates centrifuged at 16000*g* shows a protein of the correct molecular mass (46.1 kDa) which reacts with antibodies directed against CAD DHOase (Figure 4). The majority of the protein is in the supernatant although a significant amount is present in insoluble form. The DHOase deficiency of *E. coli* X7014 cells was complemented by pBZ22 (Figure 5). Activity assays of cell sonicates showed that transformed EK1104 cells had a DHOase activity ~3× higher than background while transfomed X7014 cells had an activity closer to background. The lower expression levels of the recombinant found in X7014 were confirmed by comparing the sizes of antibody-bound bands in immunoblots of sonicates from the two transformed host cells.

A purification procedure for the recombinant was developed using X7014 as host to avoid contamination by the endogenous *E. coli* DHOase (see Experimental Procedures). Later preparations of the recombinant were purified from the higher expressing EK1104 cells (Table I and Figure 6). Cells were collected by centrifugation 25 h after inoculation, and then the cell pellets were washed, sonicated, and centrifugated to remove cell debris. Ammonium sulfate was added to the sonicate supernatant to 45% saturation and incubated on ice for 30 min, and the ammonium sulfate precipitate was collected by centrifugation. The precipitate was resuspended in buffer, dialyzed, and loaded onto a DEAE-Sephacel column, and the

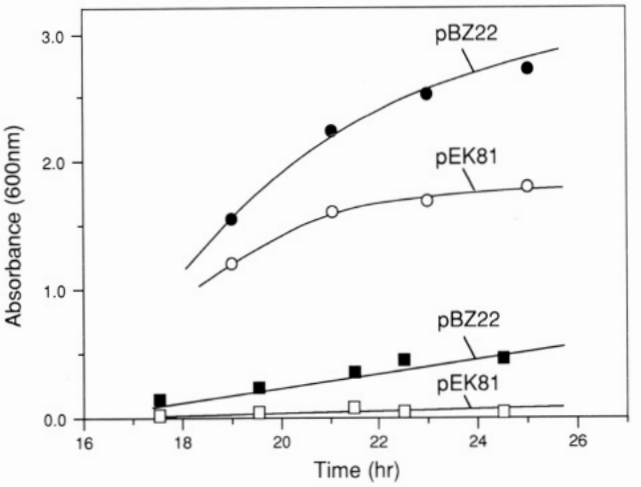

FIGURE 5: Growth curves showing complementation of DHOase-deficient *E. coli* X7014 by pBZ22. *E. coli* X7014 cells transformed with pBZ22 (●, ■) or with parent vector pEK81 (○, □) were grown on minimal media with (■, □) or with 12 μg of uracil/mL (●, ○). Cell growth was monitored by measuring the absorbance at 600 nm.

Table I: Isolation of the Recombinant Dihydroorotase Domain[a]

purification step	total protein (mg)	dihydroorotase act.[b] (μmol min^{-1} mg^{-1})
cell sonicate supernatant	44.3	0.31
dialyzed, resuspended, 45% ammonium sulfate precipitate	5.50	0.43
pooled fractions, DEAE-Sephacel column	0.35	3.86

[a] Clone pBZ22-transformed EK1104 cells were harvested 25 h after inoculation of 600 mL of minimal media containing 10 μg of uracil/mL. [b] Because the centrifuged cell sonicate and the 45% ammonium sulfate precipitate are contaminated with the endogenous *E. coli* dihydroorotase (see Figure 7), the measurements at these purification stages represent the combined activity of endogenous and recombinant proteins. The contaminating *E. coli* enzyme is removed from the preparation by fractionation on DEAE-Sephacel.

protein was eluted with a 0–0.28 M NaCl gradient (Figure 6). The recombinant protein eluted at ~0.13 M NaCl.

Approximately 550 μg of purified protein is obtained from 1 L of EK1104 cell culture. Calculations of percent yield from the first two steps of the purification procedure are complicated by the presence of endogenous DHOase from the host cells (Table I). Since the k_{cat} of the *E. coli* enyzme, 127 s^{-1} (Washabaugh & Collins, 1984), is about 40-fold higher than that of isolated CAD DHOase, 2.96 s^{-1} (Kelly et al., 1986), small amounts of contaminating enzyme could significantly contribute to activity measurements. Antibodies directed against CAD DHOase and antibodies directed against *E. coli* DHOase do not cross-react, and were used to monitor the levels of contaminating enzyme. Figure 7 shows that the amount of *E. coli* DHOase in cell sonicates is decreased by the 45% ammonium sulfate cut and that fractions eluted from the DEAE–Sephacel column are free of contamination. N-Terminal amino acid sequencing of the purified recombinant protein gave the predicted sequence (MLFVEALGQI).

The kinetic parameters obtained from a steady-state kinetic study of purified pBZ22 recombinant are compared with the parameters of the isolated domain and the intact enzyme in Table II. The turnover numbers of the recombinant protein, 2.79 ± 0.06 s^{-1}, and the domain, 2.96 ± 0.12 s^{-1} (Kelly et al., 1986), are virtually identical. The K_m values of the recombinant protein, 41.9 ± 3.5 μM, and the isolated domain, 21.8 ± 2.2 (Kelly et al., 1986), are reasonably similar, and both

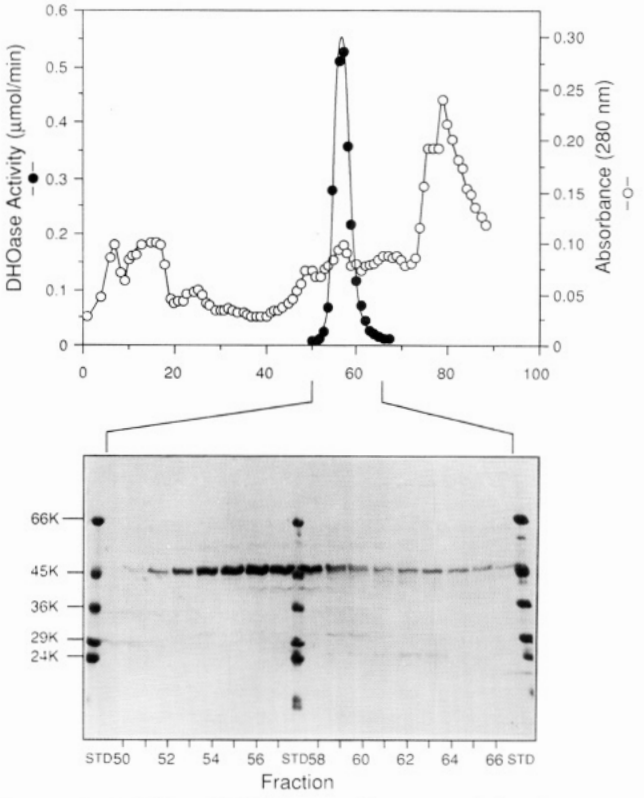

FIGURE 6: Isolation of pBZ22 recombinant protein by chromatography on DEAE-Sephacel. Fractions were eluted from a DEAE-Sephacel column loaded with the dialyzed 45% ammonium sulfate precipitate of cell sonicate (see Experimental Procedures). Upper panel: activity of fractions as measured by the colorimetric assay (●); absorbance of fractions at 280 nm (○). Lower panel: SDS gel electrophoresis of 100 μL of each fraction. Fractions 1–12 were collected during loading, fractions 13–29 were collected during the wash, and fractions 30–88 were collected during the gradient.

are significantly larger than the K_m of the intact protein, 7.38 ± 1.02 μM (Kelly at al., 1986).

DISCUSSION

The DHOase domain of the mammalian multifunctional protein CAD has been cloned and overexpressed in *E. coli*. The pBZ22 recombinant protein was positively identified by immunoblotting with domain-specific antibodies, complementation of DHOase-deficient mutants, and direct measurement of the activity of the enzyme in cell extracts. The recombinant protein was purified to homogeneity in a two-step procedure which yielded 0.55 mg from a 1-L culture. Western blotting demonstrated that the purified mammalian domain was not contaminated with *E. coli* dihydroorotase. On the basis of the activity of the purified domain and measurements of endogenous *E. coli* dihydroorotase activity, we estimated that the mammalian domain represents 3–6% of the total soluble protein.

The domain consists of 420 residues (Figure 8), extending from residue 1435 to residue 1854 in the CAD polypeptide. No residues were added from the vector at either end of the protein. The predicted molecular mass of 46.1 kDa was close to the observed value of 46 kDa. The predicted amino acid sequence of the amino end of the expressed protein was confirmed by Edman degradation, although a minor species was observed which had three residues removed by endogenous proteases. The k_{cat} of the recombinant was identical to that of the DHOase domain isolated from proteolytic digests, but the K_m value was 2-fold higher. Both the recombinant and proteolytic DHOase domains had higher K_m values and 2-fold

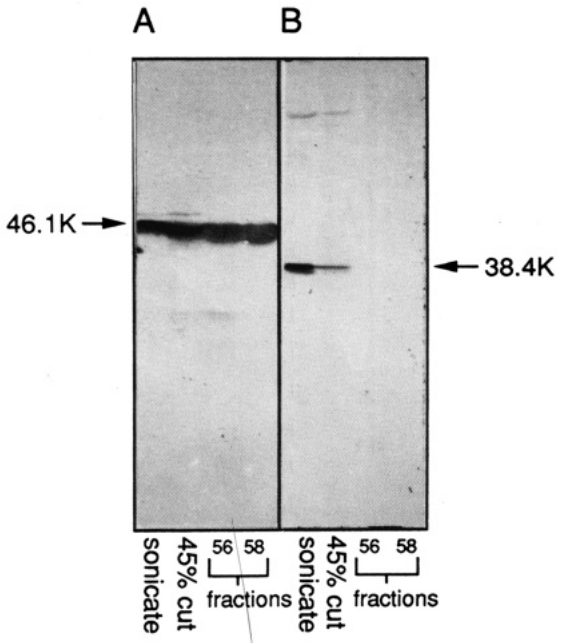

FIGURE 7: Immunoblot showing the removal of endogenous *E. coli* DHOase during purification of pBZ22 recombinant protein. Samples taken throughout the pBZ22 recombinant protein purification were electrophoresed on an SDS gel and then transferred to nitrocellulose. The blot shown in panel A was incubated with antibodies directed against CAD DHOase. The blot shown in panel B was incubated with antibodies directed against the *E. coli* DHOase. Amounts of total protein loaded are 123 μg of cell sonicate supernatant, 40.5 μg of resuspended, dialyzed 45% ammonium sulfate precipitate, and 5-μg fractions from the DEAE-Sephacel column.

Table II: Kinetic Parameters of CAD and the DHOase Domain-Catalyzed Formation of Carbamyl Aspartate from Dihydroorotate

protein	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})
CAD[a]	7.38 ± 1.02	1.52 ± 0.09	6.15 ± 0.36
proteolytic fragment[b]	21.8 ± 2.2	4.03 ± 0.16	2.96 ± 0.12
recombinant DHOase domain	41.9 ± 3.5	3.63 ± 0.08	2.79 ± 0.06

[a] Data from Mally et al. (1981). [b] Data from Kelly et al. (1986).

lower k_{cat} values than those determined for the dihydroorotase activity of intact CAD, suggesting that there may be interactions with other regions of the parent polypeptide which optimize the active conformation of the domain. This clone will be useful for mutagenesis and mechanistic studies of mammalian dihydroorotase.

By contrast, pBZ28, the first clone we obtained (Figure 8), lacked catalytic activity. The clone, which consisted of 401 residues (1404–1795), was constructed using the restriction sites that most closely bracketed the core domain (1456–1789) defined in the sequencing study (Simmer et al., 1990b). It was 34 residues longer on the amino end and 51 residues shorter on the carboxyl end than the active recombinant. The clone, which was expressed at very high levels in *E. coli*, was found initially in inclusion bodies, and could be solubilized and refolded into a soluble, stale, compactly folded, but inactive species. Refolding experiments indicated that the catalytically active proteolytic fragment could be unfolded and refolded with the restoration of catalytic activity. A zinc blotting technique that uses $^{65}ZnCl_2$ to detect zinc binding proteins immobilized on nitrocellulose (Schiff et al., 1988) was used to analyze the proteins. Our preliminary experiments show that both the CAD proteolytic DHOase and the solubilized pBZ28 recombinant protein bind zinc, although the recombinant exhibits weaker binding that the proteolytic fragment (B. H. Zimmermann and D. R. Evans, unpublished observations).

Several explanations could account for the lack of activity of the shorter clone. Residues required for substrate binding or catalysis, or structural elements necessary for the formation of the active site, may be contained within the missing sequence. Alternatively, the truncated clone may lack the ability to fold into the active conformation. It may be that the residues added from the vector on the amino and carboxyl terminals prevent correct folding. While we cannot decide between these possibilities, a comparison of the aligned sequences of this region of the multifunctional proteins from hamster, drosophila, and dictyostylium revealed low sequence identity, and very few conserved amino acids which could be considered candidates for active-site residues.

Our results are consistent with those reported by Musmanno and co-workers (Musmanno et al., 1991). Although the deletion clones constructed by these authors contained variable amounts of other sequences (Figure 8), they clearly showed that maximum dihydroorotase activity was obtained when the DHOase coding sequence extended beyond the carboxyl end of the core domain (Figure 8). A shorter clone, pCH4, extended only to residue 1793, and had minimal activity. Their results also indicated that the region of the CAD polypeptide specifying the dihydroorotase begins close to the amino end of the core domain. This result was confirmed using a recently constructed clone, pLKD1, that is expressed in mammalian cell (J. R. Davidson and R. S. Jamison, personal communication). The purification and measurement of kinetic parameters of these recombinant proteins will aid in making a direct comparison with the purified pBZ22 recombinant protein reported here.

While the precise beginning and end of the clone constructed by Williams and co-workers (Wiliams et al., 1990) was not specified, it was designed to mimic the active proteolytic fragment produced by elastase digestion. Their results indicated that the elastase fragment ended at residue 1800 on the carboxyl end, a result which does not agree with our previous findings which showed that a peptide beginning at 1811 could be isolated from exhaustive tryptic digests of the fragment (Kim et al., 1992) (Figure 8). Thus, their clone probably lacked sequences at the amino end necessary for activity.

Taken together, these studies help define the sequences encoding the functional DHOase domain. Partial sequencing of the functional fragment isolated from proteolytic digests indicates that the domain begins at 1456 (Kelly et al., 1986; Williams et al., 1990). While there are conflicting results regarding the location of the carboxyl end of the fragment, kinetic studies of the purified recombinant protein encoded by pBZ22 show that it is fully functional. Thus, the coding sequence must end at or before residue 1846, but after 1795, the carboxyl end of the active and inactive recombinants encoded by pBZ22 and pBZ28, respectively. Detection of DHOase activity in cell extracts of pLKD1-transfected mammalian cells is in agreement with this conclusion (J. R. Davidson and R. S. Jamison, unpublished results). Experiments are planned to more precisely define the carboxyl end of the domain. Although the sequence of the monofunctional dihydroorotase from *Clostridium oroticum* has not yet been determined, it is interesting that this protein is much larger, 55 kDa, than either the *E. coli* or the yeast dihydroorotase.

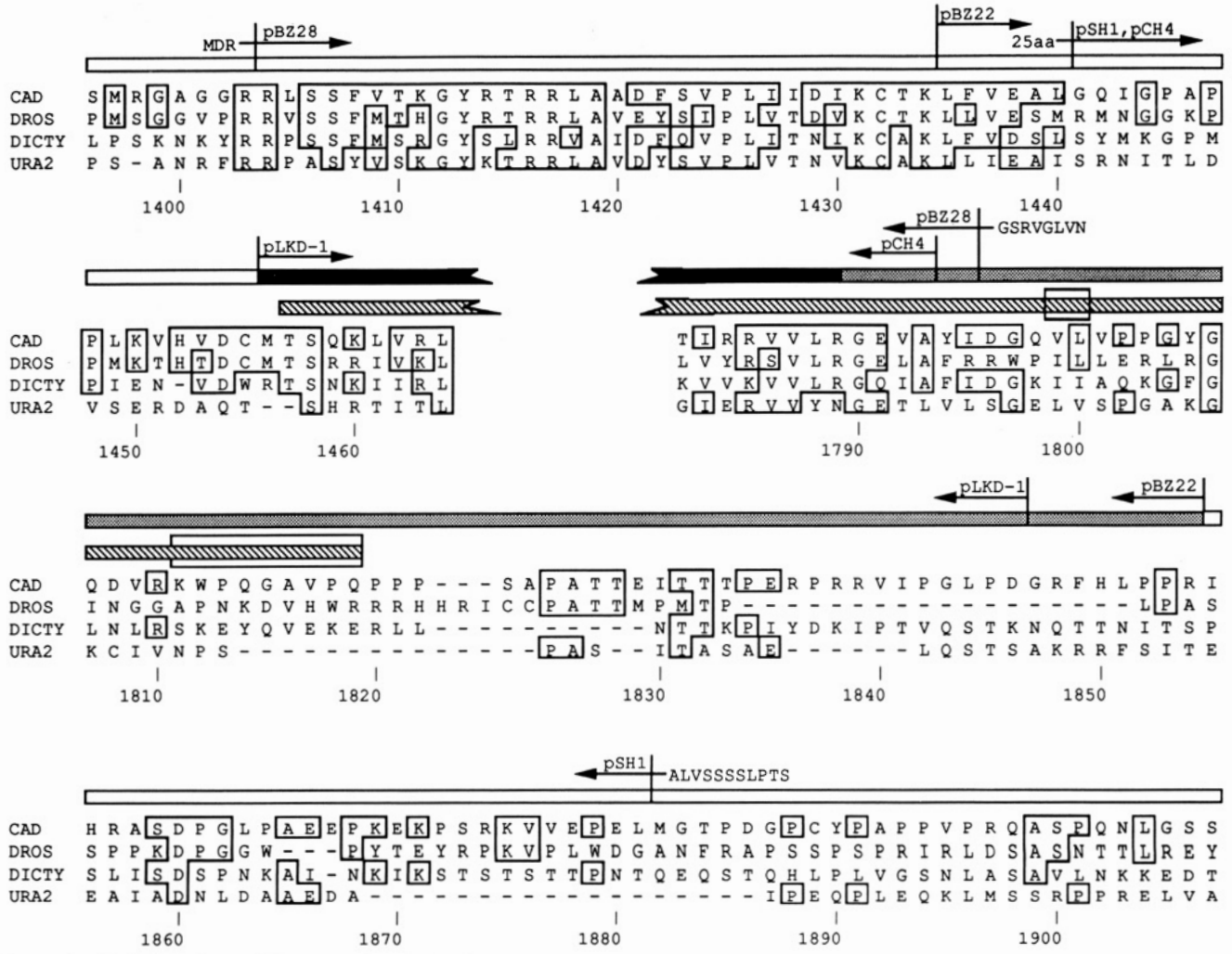

FIGURE 8: Positions of recombinant proteins in the dihydroorotase region of hamster CAD. The amino and carboxyl ends of recombinants are indicated by a vertical line and arrow above the sequence. Additional vector sequences are shown next to the name of each clone. Recombinants pBZ28 (this work) and pCH4 (Musmanno et al., 1991) are catalytically inactive, while recombinants pBZ22 (this work) and pSH1 (Musmanno et al., 1991) are active, as is pLKD-1 (J. R. Davidson and R. S. Jamison, unpublished data), a recombinant expressed in a mammalian system. The cross-hatched bar shows the minimum size of the DHOase fragment obtained from an elastase digestion of CAD, on the basis of the amino terminal found by Williams and co-workers (Williams et al., 1990), and the location of a peptide (boxed region, 1811–1819) obtained from an exhaustive tryptic digest of the CAD DHOase fragment (Kim et al., 1992). The small box at residues 1799 and 1800 shows the carboxyl end of the CAD DHOase elastase fragment according to Williams and co-workers (Williams et al., 1990). Identical residues in the aligned sequences (Simmer et al., 1990b) of hamster DHOase (CAD), *Drosophila melanogaster* (DROS) (Freund & Jarry 1987), *Dictyostelium discoideum* (DICTY) (Faure et al., 1989), and yeast interdomain linker (URA2) (Nagy et al., 1989) are boxed. There appear to be numerous frame shifts in the published nucleotide sequence of *D. melanogaster* (J. P. Simmer and D. R. Evans, unpublished observations). The amino terminal of the CAD ATCase begins 11 residues downstream of the last residue shown in the figure.

It has been suggested (Freund & Jarry, 1987; Faure et al., 1989); Simmer et al., 1990b) that the two distinctly different classes of DHOase molecules arose by convergent evolution and that the enzyme associated with the multifunctional proteins evolved separately from the long interdomain segment which connects the CPSase and ATCase domains. In support of this argument, it was noted (Freund & Jarry, 1987) that the sequence of the interdomain segment of the yeast pyrimidine biosynthetic complex, which has no dihydroorotase activity, is homologous to the DHOase domain of CAD but lacks the putative catalytic and zinc binding residues (Souciet et al., 1989). A second possibility is that this region of the molecule evolved from an ancestral monofunctional protein and represents a separate functional domain (Freund & Jarry, 1987; Souciet et al., 1989; Simmer et al., 1990b). The expression of an active mammalian dihydroorotase using a clone containing only dihydroorotase coding sequence shows that the DHOase domain, while larger than expected on the basis of homology with the monofunctional proteins, is an independent, autonomously folding domain.

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