Formation of Functional Cross-Species Heterodimers of Ornithine Decarboxylase†

Andrei Osterman, Nick V. Grishin, Lisa N. Kinch, and Margaret A. Phillips*

Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75235

Received May 6, 1994; Revised Manuscript Received July 12, 1994®

ABSTRACT: The two active sites in ornithine decarboxylase (ODC) are formed at the dimer interface with Lys-69 and Cys-360 contributing to each active site from opposite monomers [Tobias, K. E., & Kahana, C. (1993) *Biochemistry 32*, 5842−5847]. To gain insight into the organization of the substrate binding site and the nature of the dimer interface, analysis of ornithine decarboxylase from two parasitic protozoa, *Trypanosoma brucei* and *Leishmania donovani*, and from mouse was undertaken. Though *T. brucei* and mouse ornithine decarboxylase share only 60% sequence identity, the cross-species heterodimers form spontaneously, as measured by the restoration of enzyme activity upon mixing inactive K69A and C360A mutant enzymes. Thus, the amino acid composition of the dimer interface is apparently highly conserved between the *T. brucei* and mouse enzymes. Cross-species heterodimers were not formed between either *T. brucei* or mouse ODC and *L. donovani* ODC. Unlike the mouse and *T. brucei* ODC, the subunits of *L. donovani* ODC are not in rapid equilibrium, and incubation with a denaturant is required to induce reassociation. Kinetic analysis of the wild-type mouse and parasite ODCs revealed differences in the substrate binding sites between the three enzymes. The substrate binding properties of the restored active site in the *T. brucei*:mouse cross-species heterodimer mimic the characteristics of the wild-type enzyme from the species which contributes the subunit with a functional Lys-69.

Polyamines are required for cell growth and differentiation. The first committed step in polyamine biosynthesis is the decarboxylation of Orn to produce putrescine, and it is catalyzed by a pyridoxal phosphate- (PLP) dependent enzyme, ornithine decarboxylase (ODC)[1] (Tabor & Tabor, 1984). ODC has generated interest as a potential drug target for the treatment of cancer and parasitic infections (e.g., trypanosomiasis) and has been the subject of extensive investigation (McCann & Pegg, 1992). However, mechanistic and structural characterization of the eukaryotic ODCs is limited by the lack of a three-dimensional structure.

The eukaryotic ODCs are part of a multigene family including enzymes with specificity for Arg and diaminopimelate, although they are unrelated to the bacterial ODCs (Sandmeier et al., 1994). Primary structures for ODC from 14 species, including mouse and two parasitic protozoa (*Trypanosoma brucei* and *Leishmania donovani*), have been reported. Lys-69 was identified biochemically as the covalent attachment site for PLP, while Cys-360 was found to form a covalent adduct with α-difluoromethylornithine (DFMO; Lu et al., 1991; Poulin et al., 1992). Mouse ODC mutants K69A and C360A are inactive (Coleman et al., 1993; Tsirka & Coffino, 1992), but co-expression in an *in vitro* translation system restored partial enzyme activity (Tobias & Kahana, 1993; Coleman et al., 1994). Thus, based on the criteria established by Wente and Schachman for aspartate transcarbamoylase (Wente & Schachman, 1987), the two active sites of ODC are formed at the dimer interface with amino acid residues from both monomers contributing to each active site (e.g., Lys-69 is contributed from one monomer and Cys-360 from the other).

The finding that ODC has shared active sites provides a powerful approach to study the nature of the dimer interface. To get an indication of the extent of amino acid conservation at the dimer interface, we tested for the formation of cross-species heterodimers between mouse (m), *T. brucei* (tb), and *L. donovani* (ld) ODC. The cross-species mutant heterodimers of mouse and *T. brucei* ODC readily form upon mixing m-K69A with tb-C360A or upon mixing tb-K69A with m-C360A. Cross-species heterodimers do not form between *L. donovani* ODC and either of the other two enzymes.

Kinetic analysis of the host and parasite ODCs expressed as recombinant proteins in *Escherichia coli* was performed to probe for structural variation in the substrate binding site. Differences in the binding affinities for Orn and several ODC inhibitors were found between the mouse and parasite ODCs. In light of these data, characterization of the mouse: *T. brucei* cross-species active site allowed for mapping of the structural determinants of specificity differences in relation to the previously identified active site residues.

EXPERIMENTAL PROCEDURES

Materials

Substrates and inhibitors were purchased from Sigma, as was the CO_2 detection kit. Ni^{2+} resin was purchased from Qiagen. DFMO was obtained from Marion Merrel Dow, Cincinnati, OH. TEV protease was obtained from Dr. Stephen Johnston (UTSW), but is commercially available (BRL).

† This work was supported by grants to M.A.P. from the Welch Foundation (I-1257) and the Texas affiliate of the American Heart Association (93G-08S).

* Corresponding author; Phone: 214-648-3637; Fax: 214-648-2971.

® Abstract published in *Advance ACS Abstracts,* October 15, 1994.

[1] Abbreviations: ornithine decarboxylase, ODC; α-D,L-difluoromethylornithine, DFMO; α-methyl-D,L-ornithine, α-MO; mouse ODC single mutants are designated as m-K69A and m-C360A, the double mutant is designated as m-K69A/C360A, and the heterodimers resulting from mixing of the single mutants are designated as m-K69A:m-C360A; *T. brucei* (tb) and *L. donovani* (ld) ODC mutants are similarly abbreviated.

0006-2960/94/0433-13662$04.50/0

Methods

Spectrophotometric ODC Assay. The assay has been adapted from the Sigma CO_2 detection kit (Sigma 132-B) and is similar to that described for Lys decarboxylase (Scriven et al., 1988). NAD^+ production is monitored at 340 nm. Reagents are dissolved in boiled water, or when preparing reagents from lab stocks, buffer solutions are lyophilized and then dissolved in freshly boiled water. The assays were carried out in a Beckman DU650 spectrophotometer at 37 °C. The rate of NADH oxidation is linearly dependent on the concentration of ODC over a range of approximately 0.1−2.5 μg/mL. Maximum activity for mouse ODC was reported to occur at pH 7.2 (Janne & Williams-Ashman, 1971). We found that for all three enzymes, k_{cat}/K_m is insensitive to pH over a range from 7.0 to 8.2 (data not shown); the assay remains linear over a suitable pH range of 7−9. All further assays were done using the Sigma CO_2 detection kit at pH 8.0.

The assay protocol is as follows: reagents B and C (50 μL of each) are mixed into 400 μL of reagent A. The reaction is started by the addition of ODC. *Reagent A*, Sigma kit reagent A or 25 mM buffer (Tris or MOPS), pH 7−9, 0.1 mg/mL BSA, 10 mM $MgCl_2$, 0.32 mM NADH, and 2.2 mM phosphoenol pyruvate. *Reagent B*, Sigma kit reagent B or 20 mM MOPS, pH 7.2, 10 mM $MgCl_2$, 15% glycerol, 7.7 unit/mL malate dehydrogenase and 1.4 unit/mL phosphoenolpyruvate carboxykinase. *Reagent C*, 10× Orn, 25 mM DTT, and 0.5 mM pyridoxal phosphate. Substrate concentrations ranged from 0.025 to 5 mM depending on the source of the enzyme so data could be collected at concentrations above and below K_m. Michaelis−Menten parameters were calculated using the program k_{cat} (Biometallics, Inc.).

Formation of Mutant Heterodimers. The mutant proteins were mixed together at concentrations of total protein ranging from 0.002 to 0.6 mg/mL and incubated for various times before assay (buffer was either 20 mM Tris 7.5, 2 mM DTT, 20 μM pyridoxal phosphate, 0.2% Brij, or assay reagent A.) A molar concentration ratio of 1:1 for the K69A mutant to the C360A mutant was used to determine K_m. k_{cat} was determined by titrating the concentration of the K69A mutant relative to a fixed concentration of C360A as described (Figure 1; Pookanjanatavip et al., 1992). Final protein concentrations in the assay ranged from 0.5 to 6 μg/mL. For experiments with *L. donovani* ODC, the mixtures were incubated for 10 min prior to assay with 2 M deionized urea, which was the concentration found to give the maximum restored activity.

Measurement of DFMO Inhibition. DFMO (25−600 μM) was incubated with ODC (0.05−0.2 mg/mL) at 37 °C. Aliquots (5 μL) were removed at various time points (1−20 min) and diluted into 0.5 mL of assay buffer where the remaining ODC activity was measured. K_I and $t_{1/2}$ values were calculated from this data as described (Kitz & Wilson, 1962).

Expression Vector Construction. The genes encoding *T. brucei* (Phillips et al., 1987), mouse (Gupta & Coffino, 1985), and *L. donovani* (Hanson et al., 1992) ODC were cloned into the His_6-TEV vector (Parks et al., 1994). The vector directs the production of ODC from the T7 promoter as a fusion protein with an N-Terminal extension of six histidines

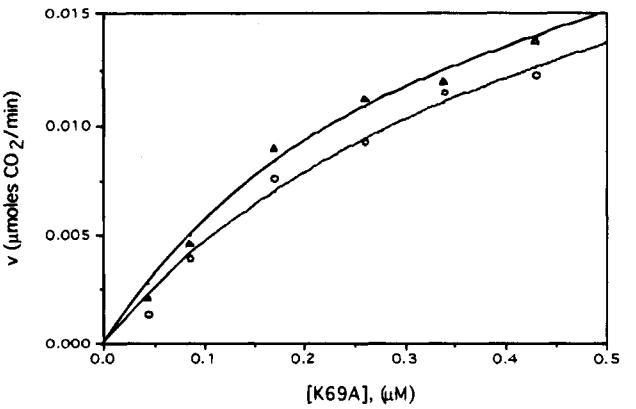

FIGURE 1: Analysis of reaction rate as a function of K69A concentration at a fixed concentration of C360A ODC (0.11 μM). (▲) tb-K69A complemented with tb-C360A; (○) tb-K69A complemented with m-C360A. Similar curves were obtained for m-K69A: m-C360A, m-K69A:tb-C360A, and ld-K69A:ld-C360A. The resultant enzyme activity was assayed at 10 mM Orn, and the data were fit to the equation $v = V_{max}[K69A]/([Cys360A] + [K69A])$ as described by Pookanjanatavip et al. (1992). The equation describes the system where monomers reassociate randomly to form dimers. The k_{cat} obtained from the curve fits are reported in Table 1.

(His-tag), which facilitates tight binding to Ni^{2+} agarose, followed by the highly specific TEV protease site. All three His-tagged enzymes were determined to be active with specific activities comparable to those published for the native and recombinant mouse (Coleman et al., 1993; Pegg & McGill, 1979; Isomoa et al., 1983) and *T. brucei* enzymes (Phillips et al., 1988). The His-tag had no effect on enzyme activity, and unless specified, His-tagged enzymes were used for analysis.

(*A*) *T. brucei ODC.* Plasmid pBCS7 (Ghoda et al., 1990) digested with *Sst*I and *Tth*III-1 was ligated to an oligonucleotide adapter, A (5′CCATGGACA3′) and B (3′TCGAGGTACCTGTA5′), inserting an *Nco*I site at the first ATG. The *NcoI/SalI* fragment from this clone was ligated to the similarly digested His_6-TEV vector. The 5′ end of *T. brucei* ODC expressed from this construct (ptbODC29) is M-H_6-AENLYFQGAMDI, where **M** is the normal start site for *T. brucei* ODC. The underlined amino acids represent the TEV protease recognition site, cleavage occurs between Q and G.

(*B*) *Mouse ODC.* The His_6-TEV vector was digested with *NcoI/SalI*; the resulting overhangs were filled in with T4 polymerase and ligated to create an *Acc*I site. The digested *AccI/Hin*dIII (blunted with T4 polymerase) vector was ligated to the 1.7-kb *TaqI/Pvu*II fragment from pOD20.7 (Gupta & Coffino, 1985). The 5′ end of mouse ODC expressed from this construct (pMOD-5) is M-H_6-AENLYFQGAMSRTMSS.

(*C*) *L. donovani ODC.* The His_6-TEV vector was digested with *NcoI/Bam*HI; the resulting overhangs were filled in with T_4 polymerase, and the ends were closed by ligation to recreate a *Bam*HI site. This modified vector was digested with *Bam*HI and *Pst*I and ligated to the 3.6-kb *BclI/Nsi*I fragment from the genomic clone of *L. donovani* ODC (Hanson et al., 1992). The 5′ end of *L. donovani* ODC expressed from this vector (pLOD11) is M-H_6AENLYFQGAMDH.

ODC Purification. Expression plasmids of wild-type or mutant ODCs were transformed into BL21/DE3 (Parks et al., 1994) cells. Expression of each enzyme was optimized

by growing the cells at several temperatures and in various media. *T. brucei* ODC remains largely soluble when cells are grown at 37 °C in LB broth, while isolation of soluble mouse and *L. donovani* ODC required growth at 23 °C in minimal medium. Cells grown to 0.8 o.d. were induced by the addition of 0.2−0.4 mM IPTG 3−12 h before harvest. The yields of soluble protein vary from 10 mg/L of bacteria for *L. donovani* and mouse ODC to 40 mg/L for *T. brucei* ODC.

Bacterial cells were lysed by high-pressure disruption into buffer A (25 mM Tris-HCl, pH 7.5, 2 mM PMSF, 5 mM β-mercaptoethanol, 50 mM NaCl, 0.02% Brij, and 0.02 mM pyridoxal phosphate) plus 1 mg/mL lysozyme. Insoluble material was removed by sedimentation in a Beckman Ti-45 rotor (150000*g* for 1 h), and the supernatant was applied to a Ni^{2+} agarose column (Qiagen). The column was washed with buffer A plus 0.5 M NaCl to remove proteins with low affinity, and then His-tagged ODC was eluted with a gradient of imidazole (0−200 mM) in buffer A. ODC-containing fractions were dialyzed, concentrated, and applied to a High-load 16/60 Superdex G-200 gel filtration column (Pharmacia) in buffer C (25 mM HEPES, pH 7.2, 2 mM DTT, 0.5 mM EDTA, and 0.02 mM pyridoxal phosphate). All three enzymes were at least 98% pure after these two steps.

Protein Determination. Samples of mouse, *T. brucei*, and *L. donovani* ODCs were submitted for amino acid analysis, and the extinction coefficients were determined from this data (within experimental error $\epsilon = 0.85$ o.d. $(mg/mL)^{-1}$ cm^{-1} for *T. brucei*, *L. donovani*, and mouse ODC). The protein concentration in all samples used for kinetic analysis was determined spectrophotometrically.

Native Size Determination for L. donovani ODC. The monomer molecular mass of *L. donovani* ODC was estimated previously to be 76 kDa (Coons et al., 1990). Gel filtration analysis on a Superose-12 column (Pharmacia) indicated that, like mouse and *T. brucei* ODC, native *L. donovani* ODC is a dimer.

Mutagenesis. Mutagenesis was performed by the standard Kunkel technique (Kunkel, 1985) in the Bluescript vector (Stratagene) using the M13 helper phage R408 (Stratagene) and the Kunkel strain BO265. The primers were as follows: *T. brucei*, K69A, 5′-TTTACGCGGTCGCATG-CAAC; C360A, 5′-GGGGTCCCACAGCTGATGGT; mouse, K69A, 5′-TACGCAGTCGCATGTAACGATAGCAGGGC-CAT; C360A, 5′-GGACCAACAGCAGATGGTCTA-GATCG; *L. donovani* ODC, K69A, 5′-TTTGCCGTG-GCAAGCAAC; C360A, 5′-GGCCCCACCGCAGATTCC-ATGGAC. The codons which have been replaced with Ala are underlined. Double mutants of K69A and C360A were then constructed for each enzyme by subcloning.

RESULTS

Substrate and Inhibitor Specificity among Host and Parasite ODCs. ODC was assayed spectrophotometrically by the coupling of Orn decarboxylation to the oxidation of NADH via the consecutive action of phosphoenolpyruvate carboxykinase and malate dehydrogenase as described in Experimental Procedures. This assay allows the reaction to be followed continuously over time providing advantages over the traditional method of CO_2 trapping (Seeley & Pegg, 1983). For ODC-catalyzed decarboxylation of Orn, the K_m for *T. brucei* and *L. donovani* ODC are 2−3- and 5-fold higher, respectively, than for mouse ODC, while k_{cat} is invariant (Table 1). The binding affinity for the substrate analog α-methylornithine (α-MO) is similar to the substrate K_m for both mouse and *L. donovani* ODC (Tables 1 and 2). For *T. brucei* ODC, the K_I for α-MO is 2-fold higher. DFMO is a time-dependent inhibitor for all three enzymes. The binding affinity for DFMO parallels the trends seen for K_m, and the half-life of inactivation ($t_{1/2}$) is similar for all three enzymes (Table 2). In contrast, the product putrescine binds to *T. brucei* ODC 2-fold more tightly than to mouse or *L. donovani* ODC. The two parasite enzymes bind putrescine with similar affinity to Orn, while mouse ODC binds Orn with 7-fold greater affinity than putrescine (Table 2).

Table 1: Kinetic Analysis of Wild-Type and Mutant Heterodimers[a]

	Wild-Type Enzymes		
	enzyme		
	mouse	*T. brucei*	*L. donovani*
K_m	0.090 ± 0.0079	0.18 ± 0.011	0.42 ± 0.039
k_{cat}	7.8 ± 0.3	7.4 ± 0.08	7.7 ± 0.2
k_{cat}/K_m	87 ± 8.4	41 ± 2.3	19 ± 1.8
	Same-Species Heterodimers		
	m-K69A:m-C360A	tb-K69A:tb-C360A	ld-K69A:ld-C360A
K_m	0.051 ± 0.0096	0.24 ± 0.035	0.35 ± 0.080
k_{cat}	9.2 ± 1.8	7.8 ± 1.0	1.2 ± 0.030
k_{cat}/K_m	180 ± 47	33 ± 6.0	3.5 ± 0.80
	Cross-Species Heterodimers		
	tb-K69A:m-C360A	m-K69A:tb-C360A	ld:tb or ld:m
K_m	0.049 ± 0.0037	0.26 ± 0.038	not formed
k_{cat}	9.1 ± 1.5	9.7 ± 2.1	
k_{cat}/K_m	190 ± 34	37 ± 9.6	

[a] K_m, mM; k_{cat}, s^{-1}, calculated per active site; k_{cat}/K_m, mM^{-1} s^{-1}. For the cross-species and same-species heterodimers, k_{cat} was calculated as described in the legend to Figure 1.

Table 2: Inhibition of Host and Parasite ODCs[a]

enzyme	α-MO K_I (mM)	putrescine K_I (mM)	DFMO	
			K_I (mM)	$t_{1/2}$ (min)
mouse	0.10 ± 0.019[d]	0.61 ± 0.058[c]	0.038 ± 0.0029[e]	2.2 ± 0.07
T. brucei	0.48 ± 0.047	0.35 ± 0.043	0.13 ± 0.038[b]	1.6 ± 0.3
L. donovani	0.47 ± 0.018	0.61 ± 0.032	0.17 ± 0.07	2.7 ± 0.4

[a] $t_{1/2}$ is the calculated half-life of inactivation at infinite inhibitor concentration. The results are similar to previous reports for the following references: [b] Phillips et al. (1988); [c] Janne and Williams-Ashman (1971); [d] Bey et al. (1978); [e] Metcalf et al. (1978).

Formation and Kinetic Analysis of Same-Species and Cross-Species Mutant Heterodimers. To test for the formation of cross-species heterodimers, the single and double mutants (if listed) of K69A and C360A for mouse (m-K69A, m-C360A, and m-K69A/C360A). *T. brucei* (tb-K69A, tb-C360A, and tb-K69A/C360A) and *L. donovani* (ld-K69A and ld-C360A) ODC were made, expressed, and purified as described in Experimental Procedures. Enzyme activity will be restored upon mixing of the inactive K69A mutant from one species with the inactive C360A mutant of another, only in the event that functional cross-species heterodimers form. Additionally, if the cross-species heterodimers form, the double mutant of one species would be expected to inhibit the wild-type enzyme of the other. The experiments

characterizing the same-species and cross-species heterodimers are described below.

Same-Species Mutant Heterodimers (m-K69A:m-C360A, tb-K69A:tb-C360A, and ld-K69A:ld-C360A). The specific activities of the K69A mutants for all three enzymes are no greater than 0.1% of wild-type activity. The specific activity of the C360A mutants range from 1.5% for *T. brucei* and mouse ODC to 3% of wild-type activity for *L. donovani* ODC. The values obtained for the mouse ODC mutants are in agreement with previous reports (Coleman et al., 1993).

Dissociation of ODC dimers into monomers is reportedly promoted by salt concentrations above 0.25 mM in the absence of substrate (Solano et al., 1985), suggesting that the formation of the mutant heterodimers would not require co-translation or a denaturation/renaturation cycle as required for other proteins with shared active sites (e.g., aspartate transcarbamoylase; Wente & Schachman, 1987). After the mixing of either mouse or *T. brucei* K69A mutants with the same-species C360A mutant, the exchange of monomers between the two mutant dimers occurs spontaneously (in less than 5 min) even at low ionic strength in agreement with a recent report on mouse ODC (Coleman et al., 1994). In contrast, *L. donovani* ODC monomers did not rapidly exchange between dimers. Consistent exchange of monomers between ld-K69A and ld-C360A, as monitored by the restoration of ODC activity, can be obtained by incubation of the mixture in the presence of 2 M urea prior to reassociation in assay buffer.

For each mutant, the protein concentration used in the mixture was too low for enzymatic activity to have been detected if the mutant alone was to be assayed as a homodimer in a separate experiment. Therefore, the enzymatic activity observed in the mixture is due solely to the formation of the mutant heterodimer. For 1:1 molar mixtures of m-K69A:m-C360A and tb-K69A:tb-C360A, the specific activity is restored to approximately 25% of wild-type levels, as expected by the theoretical model (Wente & Schachman, 1987) and consistent with previous reports for mouse ODC (Tobias & Kahana, 1993; Coleman et al., 1994). In contrast, for a 1:1 molar mixture of ld-L69A:ld-C360A, activity is only partially restored. The inability to restore the full level of expected activity is likely due to incomplete dissociation of the subunits in 2 M urea.

The k_{cat}'s for the restored single active site in the same-species mutant heterodimers were calculated by titrating the concentration of K69A mutant relative to a fixed concentration of C360A mutant as described (Pookanjanatavip et al., 1992). Plots of the restored activity vs the concentration of K69A used in this calculation are displayed in Figure 1. For the mouse or *T. brucei* mutant heterodimers, k_{cat} for the single active site is identical to the wild-type k_{cat} (Table 1). For *L. donovani* mutant heterodimers, k_{cat} is only 16% of the wild-type level. K_m for Orn decarboxylation catalyzed by m-K69A:m-C360A is 2-fold lower than for wild-type mouse ODC, while the K_m's for tb-K69A:tb-C360A and ld-L69A:ld-C360A are not significantly different from the K_m observed for the wild-type enzymes (Table 1).

Cross-Species Mutant Heterodimers (m-K69A:tb-C360A and tb-K69A:m-C360A). The K69A mutants from mouse and *T. brucei* were mixed with the C360A mutants from the opposite species as described above for the same-species mutant heterodimers. Both m-K69A:tb-C360A and tb-K69A:m-C360A exhibit ODC activity, demonstrating that the cross-species mutant heterodimers readily form. For these mixtures, enzyme activity is restored to levels comparable to the same-species mutant heterodimers (Table 1 and Figure 1), while no activity is detected in control mixtures of m-K69A:tb-K69A and tb-C360A:m-C360A. Further evidence for the formation of cross-species heterodimers was obtained by mixing the double mutant of mouse (m-K69A/C360A) or *T. brucei* ODC (tb-K69A/C360A) with the wild-type enzyme of the other species. In both cases, the presence of the double mutant reduces the expected activity to 25% of the activity if all of the protein in the mixture was wild-type, similar to data reported for the inhibition of mouse ODC with m-K69A/C360A (Tobias & Kahana, 1993; Coleman et al., 1994).

Interestingly, K_m for Orn decarboxylation catalyzed by the cross-species mutant heterodimers is dependent on the origin of the subunit which contributed the functional Lys-69. In the mixture which contained a mouse ODC monomer with a functional Lys-69 (tb-K69A:m-C360A), K_m for Orn is similar to that observed for m-K69A:m-C360A and for wild-type mouse ODC. Likewise, K_m measured for m-K69A:tb-C360 is identical to K_m measured for tb-K69A:tb-C360 and for wild-type *T. brucei* ODC (Table 1).

Cross-species heterodimers do not form between mouse or *T. brucei* ODC mutants and *L. donovani* ODC mutants. No detectable activity is restored upon mixing ld-K69A with m-C360A or tb-C360A or upon mixing ld-C360A with m-K69A or tb-K69A, even after prolonged incubations (up to 12 h) or in the presence of 2 M urea. Consistent with this result, neither the double mutants of mouse (m-K69A/C360A) nor *T. brucei* ODC (tb-K69A/C360A) are able to inhibit the activity of wild-type *L. donovani* ODC.

DISCUSSION

Recent studies have demonstrated that the ODC active sites are formed at the dimer interface (Tobias & Kahana, 1993; Coleman et al., 1994). By extending the study of the ODC shared active sites to enzymes from two parasitic protozoa, *T. brucei* and *L. donovani*, we were able to gain additional insight into the organization of active site and of the dimer interface in this enzyme family.

Mutation of K69A and of C360A inactivated *T. brucei* and *L. donovani* ODC to a similar extent as reported for mouse ODC (Tobias & Kahana, 1993). Mixing of tb-K69A with tb-C360A restored enzyme activity as expected for an enzyme with a shared active site. Like mouse ODC (Coleman et al., 1994), *T. brucei* ODC subunits are in rapid equilibrium; the exchange of subunits from homodimers to heterodimers occurred spontaneously in less than 5 min. In contrast, the exchange of subunits between ld-K69A and ld-C360A required incubation with 2 M urea. The observation that urea is required for the dissociation of *L. donovani* subunits suggests that *L. donovani* ODC forms a more stable dimer than the *T. brucei* or mouse enzymes. *L. donovani* ODC has an unusual 24 kDa N-terminal extension that is not found in any other ODC (Hanson et al., 1992). This extension is most likely responsible for the increased stability of the dimeric structure.

We found that *T. brucei* and mouse ODC can form cross-species heterodimers, while cross-species heterodimers are not formed between *L. donovani* ODC and either *T. brucei* or mouse ODC. Formation of cross-species heterodimers

was shown in two ways. First, the double mutant of either mouse (m-K69A/C360A) or *T. brucei* ODC (tb-K69A/C360A) inhibits the wild-type enzyme of the other species, demonstrating that the mouse and *T. brucei* monomers are capable of physical interaction. Second, enzyme activity was restored upon mixing inactive tb-K69A with inactive m-C360A or upon mixing m-K69A with tb-C360A, and the level of restored activity was identical to that observed for the same-species mutant heterodimers. Thus, the mouse and *T. brucei* monomers not only interact but also interact to form a fully functional dimeric enzyme.

The ability of the mouse and *T. brucei* ODC monomers to associate into dimers provided a unique opportunity to study substrate binding characteristics of the shared active site in comparison to the wild-type enzymes. Differences in the binding affinity for substrate and inhibitors were found between the mouse and parasite enzymes, indicating that there are structural differences in their binding pockets. Mouse ODC binds Orn more tightly than either parasite enzyme (Table 1). The binding affinity for the substrate analog, α-MO, and for the suicide inhibitor, DFMO, parallels the same trends observed in Orn binding (Table 2). Additionally, K_I for α-MO for both mouse and *L. donovani* ODC are nearly identical to K_m for Orn (Table 2), suggesting that K_m reflects the actual dissociation constant for enzyme binding to substrate ($K_m = K_s$).

Interestingly, inhibition by putrescine did not follow the same trends. Mouse ODC has one-seventh the affinity for putrescine as for Orn, while putrescine binds to both *T. brucei* and *L. donovani* ODC with nearly the same affinity as Orn (Table 2). Thus, product inhibition maybe a more potent form of cellular regulation for the parasite enzymes than for host ODC, consistent with the findings that mammalian ODC is highly regulated by other mechanisms (e.g., intracellular turnover; Li & Coffino, 1994), while the parasite enzymes are not (Phillips et al., 1987; Bass et al., 1992). Thus, *in vivo*, putrescine analogs may be more toxic to the parasite than the host. Additionally, mouse ODC binds Orn, α-MO, and DFMO more tightly than the parasite enzymes, while the affinity for putrescine is similar among them. These data suggest the specificity differences between the host and parasite ODCs may arise from differences in the nature of the interactions between ODC and the α-carboxylate.

The K_m's and k_{cat}'s measured for the decarboxylation of Orn by the cross-species heterodimers are nearly identical to the wild-type values, demonstrating that the cross-species active site structure is not disrupted with respect to either substrate binding or the chemistry of decarboxylation. However, the ODC isoform which contributes the subunit with the functional Lys-69 dictates if the substrate binding properties of the cross-species heterodimer will be those of mouse or of *T. brucei* wild-type ODC (Table 1). Minimally, these data suggest that the structural features on the enzyme which account for the differences in Orn binding between mouse and *T. brucei* ODC are contributed to the active site from the same monomer as Lys-69. Taken together with the observations on Orn vs putrescine binding discussed above, the functional groups in the ODC active site which interact with the α-carboxylate are likely to be contributed to the active site from the same subunit as Lys-69.

The formation of cross-species heterodimers also provides information about the similarity of the dimer interface between mouse and *T. brucei* ODC. Amino acid residues identified by structural analysis at the dimer interface of triosephosphate isomerase are poorly conserved when comparing sequences from different species (Lolis et al., 1990). Thus, it is surprising that despite the fact that mouse and *T. brucei* ODC share only 60% overall sequence similarity (Phillips et al., 1987), cross-species mutant heterodimers not only form but are active. The finding that the k_{cat}'s for decarboxylation of Orn by the cross-species heterodimers are unaltered from the wild-type values suggests that the dissociation constant for the mouse:*T. brucei* dimer is close to that of the wild-type enzymes (assuming the wild-type enzyme provides a measure of the maximum catalytic efficiency of the system). Any advantage for the formation of the homodimers (C360A or K69A) in the mixture would shift the product ratio away from the formation of heterodimers, and the observed k_{cat} would be lower than for the wild-type enzymes. Thus, the specific interactions between the monomers in the dimer must be quite conserved between *T. brucei* and mouse ODC. Two other examples of cross-species heterodimers (discussed below) have been reported that support this conclusion.

While cross-species heterodimers between mammalian triosephosphate isomerases (the dimer interface shares 84% sequence identity) form readily, heterodimers did not form as effectively between yeast and the mammalian enzymes (the dimer interface shares only 70% identity; Sun et al., 1992). Active cross-species heterodimers of thymidylate synthase form between enzymes from two different bacterial sources sharing an overall sequence identity of 60% (Greene et al., 1993). However, sequence identity at the dimer interface was determined from the X-ray structure to be 80%. Cross-species heterodimers of thymidylate synthase could not form between species that shared only 60% identity at the interface. The results on triosephosphate isomerase and thymidylate synthase suggest that the ODC dimer interface between *T. brucei* and mouse is likely to share sequence identity higher than the overall sequence identity between them.

The finding that *L. donovani* ODC does not form cross-species heterodimers may have two structural origins: (1) *L. donovani* ODC shares only 40% sequence identity with the other two enzymes (in the common domain excluding the N-terminal extension) as compared to 60% sequence identity between mouse and *T. brucei* ODC, and (2) the N-terminal extension may contribute to the dimer stability of the *L. donovani* homodimer, providing an energetic advantage to the re-formation of the homodomer over the cross-species heterodimer upon renaturation.

In summary, we found that cross-species heterodimers were formed between mouse and *T. brucei* ODC but not between *L. donovani* ODC and either of the other two enzymes. The formation of the *T. brucei*:mouse heterodimer allowed us to study the origin of differences in substrate binding affinities between the host and parasite enzymes. Our data suggest that these differences arise from amino acid variations at the site of interaction with the substrate α-carboxylate and that the residues which are involved in this binding interaction are contributed from the same subunit as Lys-69. While residues which form interactions that stabilize the dimer interface are apparently well conserved between mouse and *T. brucei* ODC, large differences are likely to exist between the composition of the mouse or *T. brucei* and *L. donovani* ODC dimer interfaces. Further

identification of the structural basis for differences in the binding pocket and in the dimer interface will aid in the design of parasite-specific ODC inhibitors.

ACKNOWLEDGMENT

We would like to thank Dr. Buddy Ullman and Dr. Phil Coffino for kindly providing us with the *L. donovani* and mouse ODC clones, respectively, and Dr. Stephen Johnston for providing the His_6-TEV vector and purified TEV protease. DFMO was a generous gift from Merrel Dow, Cincinnati, OH.

REFERENCES

Bass, K. E., Sommer, J., Cheng, Q. L., & Wang, C. C. (1992) *J. Biol. Chem. 267*, 11034−11037.

Bey, P., Danzin, C., Dorsselaer, V. V., Mamont, P., Jung, M., & Tardif, C. (1978) *J. Med. Chem. 21*, 50−55.

Coleman, C. S., Stanley, B. A., & Pegg, A. E. (1993) *J. Biol. Chem. 268*, 24572−24579.

Coleman, C. S., Stanley, B. A., Viswanath, R., & Pegg, A. E. (1994) *J. Biol. Chem. 269*, 3155−3158.

Coons, T., Hanson, S., Bitonti, A. J., McCann, P. P., & Ullman, B. (1990) *Mol. Biochem. Parasitol. 39*, 77−90.

Ghoda, L., Phillips, M. A., Bass, K. E., Wang, C. C., & Coffino, P. (1990) *J. Biol. Chem. 265*, 11823−11826.

Greene, P. J., Maley, F., Pedersen-Lane, J., & Santi, D. V. (1993) *Biochemistry 32*, 10283−10288.

Gupta, M., & Coffino, P. (1985) *J. Biol. Chem. 260*, 2941−2944.

Hanson, S., Adelman, J., & Ullman, B. (1992) *J. Biol. Chem. 267*, 2350−2359.

Isomoa, V. V., Pajunen, A. E. I., Bardin, C. W., & Janne, O. A. (1983) *J. Biol. Chem. 258*, 6735−6740.

Janne, O. A., & Williams-Ashman, H. G. (1971) *J. Biol. Chem. 246*, 1725−1732.

Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245−3249.

Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488.

Li, X., & Coffino, P. (1994) *Mol. Cell. Biol. 14*, 87−92.

Lolis, E., Alber, T., Davenport, R. C., Rose, D., Hartman, F. C., & Petsko, G. A. (1990) *Biochemistry 29*, 6609−6618.

Lu, L., Stanley, B. A., & Pegg, A. E. (1991) *Biochem. J. 277*, 671−675.

McCann, P. P., & Pegg, A. E. (1992) *Pharmacol. Ther. 54*, 195−215.

Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, P., & Vevert, J. P. (1978) *J. Am. Chem. Soc. 100*, 2551−2553.

Parks, P. D., Leuther, K. K., Howard, E. D., Johnston, S. A., & Dougherty, W. G. (1994) *Anal. Biochem. 216*, 413−417.

Pegg, A. E., & McGill, S. (1979) *Biochim. Biophys. Acta 568*, 416−427.

Phillips, M. A., Coffino, P., & Wang, C. C. (1987) *J. Biol. Chem. 262*, 8721−8727.

Phillips, M. A., Coffino, P., & Wang, C. C. (1988) *J. Biol. Chem. 263*, 17933−17941.

Pookanjanatavip, M., Yuthavong, Y., Greene, P. J., & Santi, D. V. (1992) *Biochemistry 31*, 10303−10309.

Poulin, R., Lu, L., Ackerman, B., Bey, P., & Pegg, A. E. (1992) *J. Biol. Chem. 267*, 150−158.

Sandmeier, E., Hale, T. I., & Christen, P. (1994) *Eur. J. Biochem. 221*, 997−1002.

Scriven, F., Wlasichuk, K. B., & Palcic, M. M. (1988) *Anal. Biochem. 170*, 367−371.

Seeley, J. E., & Pegg, A. E. (1983) *Methods Enzymol. 94*, 158−161.

Solano, F., Penafiel, R., Solano, M. E., & Lozano, J. A. (1985) *FEBS Lett. 190*, 324−328.

Sun, A.-Q., Yuksel, K. U., & Gracey, R. W. (1992) *J. Biol. Chem. 267*, 20168−20174.

Tabor, C. W., & Tabor, H. (1984) *Annu. Rev. Biochem. 53*, 749−790.

Tobias, K. E., & Kahana, C. (1993) *Biochemistry 32*, 5842−5847.

Tsirka, S., & Coffino, P. (1992) *J. Biol. Chem. 267*, 23057−23062.

Wente, S. R., & Schachman, H. K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 31−35.