The Two Monofunctional Domains of Octameric Formiminotransferase−Cyclodeaminase Exist as Dimers†

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ABSTRACT: Formiminotransferase−cyclodeaminase is a bifunctional enzyme arranged as a circular tetramer of dimers that exhibits the ability to efficiently channel polyglutamylated folate between catalytic sites. Through deletion mutagenesis we demonstrate that each subunit consists of an N-terminal transferase active domain and a C-terminal deaminase active domain separated by a linker sequence of minimally eight residues. The full-length enzyme and both isolated domains have been expressed as C-terminally histidine-tagged proteins. Both domains self-dimerize, providing direct evidence for the existence of two types of subunit interfaces. The results suggest that both the transferase and the deaminase activities are dependent on the formation of specific subunit interfaces. Because channeling is not observed between isolated domains, only the octamer appears able to directly transfer pentaglutamylated intermediate between active sites.

The bifunctional enzyme formiminotransferase−cyclodeaminase (FTCD)[1] catalyzes two sequential reactions in the histidine degradation pathway. This enzyme transfers a one-carbon unit from formiminoglutamate to tetrahydrofolate, thus serving as an additional entry point to the folate pool in liver [for a review, see Shane and Stokstad (1984)]. Although electron microscopy has shown that porcine liver FTCD consists of eight identical subunits arranged to form a circular octamer (Beaudet & MacKenzie, 1976), the enzyme is more aptly described as a tetramer of dimers (MacKenzie *et al.*, 1980). A series of denaturation and renaturation experiments indicated that octameric FTCD can dissociate to form two distinct types of monofunctional dimers (Findlay & MacKenzie, 1987; 1988). These dimers display different catalytic activities and affinities for substrate and were proposed to isolate different subunit interfaces. According to this hypothesis, the tetramer of dimers includes two types of subunit interaction which must be maintained for concurrent expression of both the transferase and deaminase activities.

FTCD can directly transfer polyglutamylated formimino-tetrahydrofolate between the transferase and deaminase active sites (MacKenzie, 1979; MacKenzie & Baugh, 1980). As first demonstrated by MacKenzie (1979), the efficiency of channeling is dependent on the length of the polyglutamate tail attached to the folate. The specificity of the enzyme for pentaglutamate led MacKenzie and Baugh (1980) to suggest that the polyglutamate chain may act to anchor the substrate to the octamer while allowing the pteroyl moiety to move between the two types of catalytic sites. Further support for this model was realized when Paquin *et al.* (1985) observed only four high-affinity polyglutamate binding sites per octamer. This, in combination with other kinetic experiments, indicated the existence of only one polyglutamate binding site per pair of transferase−deaminase active sites. Findlay and MacKenzie (1988) observed that after dissociation of the FTCD octamer, the polyglutamate specificity was retained by the deaminase active dimer.

Multifunctional enzymes are thought to be composed of different modules responsible for specific binding or catalytic functions (Wetlaufer, 1973; Rossman & Argos, 1981). As the transferase and deaminase activities are kinetically independent and a transferase active fragment can be isolated after limited proteolysis (Tabor & Wyngarden, 1959; MacKenzie, 1979; MacKenzie *et al.*, 1980), it seemed possible that the two activities reside within separable domains. Therefore deletion mutagenesis of the FTCD cDNA (Murley *et al.*, 1993) was used to isolate separate transferase and deaminase active domains and to delineate the interdomain region. These domains were characterized in terms of their quaternary structure and ability to channel the product of the transferase activity to the deaminase active site.

EXPERIMENTAL PROCEDURES

Restriction and DNA-modifying enzymes were obtained from Bethesda Research Laboratories and New England Biolabs. All reagents for sequencing DNA were supplied by USB Corp. Nitrocellulose membranes were from Schleicher and Schuell. ^{125}I-Labeled protein A and

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[1] Abbreviations: FTCD, formiminotransferase−cyclodeaminase; $FTCDH_6$, hexahistidine-tagged formiminotransferase−cyclodeaminase; FTH_6, hexahistidine-tagged formiminotransferase domain; CDH_6, hexahistidine-tagged cyclodeaminase domain; H_4PteGlu, tetrahydrofolate; FIGLU, formiminoglutamate; Ni-NTA, nickel-chelated nitrilotriacetic acid matrix; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; MOPS, 4-morpholinopropanesulfonic acid; kDa, kilodalton(s); kb, kilobase(s); TFA, trifluoroacetic acid; LC/MS, liquid chromatography/mass spectroscopy; HPLC, high-performance liquid chromatography; ESI-MS, electrospray ionization mass spectroscopy.

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[^{35}S]dATP were purchased from Amersham Corporation. Oligonucleotides were synthesized and purified by the Sheldon Biotechnology Centre, McGill University, or General Synthesis and Diagnostics.

DEAE-Sepharose and the Superose 6 HR 10/30 column were purchased from Pharmacia. Ni-NTA matrix came from Quiagen, and DEAE53 was from Whatman. Folic acid and formimino-L-glutamic acid were from Sigma. Pteroylpenta-γ-glutamic acid was from Dr. B. Schircks Laboratories (Jona, Switzerland). All other chemicals were of reagent grade.

Folic acid and pteroylpenta-γ-glutamic acid were converted to the corresponding (6*S*)-tetrahydro derivatives and purified on DEAE-cellulose as described by MacKenzie and Baugh (1980). 5-Formimino-H_4PteGlu was prepared enzymatically as described previously (Paquin *et al.*, 1985) except that purified recombinant transferase domain was used to catalyze its synthesis. Unless otherwise noted, molecular biology techniques were performed as outlined in Sambrook *et al.*, (1989).

Bacterial Strains and Plasmids. pBke-Cml is a previously described FTCD expression vector (Murley *et al.*, 1993). pET23d (Novagen) was the source of the C-terminal hexahistidine tag. *Escherichia coli* strain BL21/DE3 was employed for expression of recombinant proteins, DH5α was used for cloning purposes, and CJ236 was used to produce uracil-containing single-strand DNA templates for site-directed mutagenesis (Kunkel *et al.*, 1987).

Preparation of N-Terminal and C-Terminal Deletion Constructs. The FTCD expression plasmid pBke-Cml was used to construct vectors that would express separately the transferase and deaminase domains. The terminology used to describe these vectors incorporates the amino acid numbering of the full-length FTCD. The N-terminal fragment represented by FT327-1 is a protein starting at the FTCD initiator methionine and terminating after residue 327 plus one non-related amino acid. CD333 represents a protein beginning at FTCD residue 333 and ending at the normal stop codon after residue 541. The plasmids expressing these fragments are pBke-FT327-1 and pBke-CD333.

FTCD proteins truncated at the C-terminus were generated by one of two strategies. FT318-1, FT321-3, FT327-1, FT331, FT335-2, and FT340-2 were produced through Exo/S1 deletion mutagenesis (Henikoff, 1987) and insertion of translational stop codons in all three reading frames. Dideoxy sequencing was used to identify constructs which terminated at the desired positions (Sanger *et al.*, 1977). To construct FT322 and FT325, stop codons were introduced immediately following the codon for residue 322 or 325 by oligonucleotide-directed mutagenesis (Kunkel *et al.*, 1987) of pBke-Cml. Clones containing the desired mutations were identified by dideoxy sequencing.

To create constructs expressing the C-terminal fragments (CD), pBke-Cml was re-engineered to remove the intervening coding sequence between the initiating ATG and the desired initiating residue, using a strategy previously described for the construction of pBke-Cml (Murley *et al.*, 1993). The new translation initiation site was confirmed by dideoxy sequencing.

Construction of Plasmids Expressing Histidine-Tagged FTCD and Domains. Hexahistidine tags (H_6) were engineered onto the C-terminal ends of FTCD and the N- and C-terminal domains. To produce $FTCDH_6$, we first replaced the FTCD stop codon in pBke-Cml with an *Xho*I site, creating pBke-Cml*Xho*I-1. Then, using standard molecular biology techniques, an *Xho*I/*Esp*I fragment encoding the hexahistidine tag from pET23d was inserted in frame immediately after the new *Xho*I site. Thus pBke-$FTCDH_6$ expresses an FTCD with eight additional amino acids attached to its C-terminus: leucine, glutamate, and six additional histidines. To construct pBke-CDH_6, which expresses a histidine-tagged C-terminal domain (CDH_6), a fragment encoding the N-terminus of pBke-$FTCDH_6$ was replaced with the corresponding fragment from pBke-CD333. pBke-$CD339H_6$ was constructed in a similar manner.

In order to construct pBke-FTH_6, a vector expressing the histidine-tagged N-terminal domain (FTH_6), we first introduced an *Xho*I site into pBke-Cml immediately after codon 328. The cDNA following this *Xho*I site was replaced with an in-frame *Xho*I/*Bfa*I fragment from pET23d, which provided the same eight amino acids and stop codon described above.

Western Analysis. BL21/DE3 expressing the N- and C-terminal deletion mutants were grown and harvested as previously described (Murley *et al.*, 1993). Frozen *E. coli* cell pellets were suspended in approximately 3 vol of sonication buffer I [0.1 M potassium phosphate (pH 7.3), 25 mM 2-mercaptoethanol, 1 mM benzamidine, and 1 mM PMSF] and sonicated for a total of 10 × 15 s. Following centrifugation, the insoluble fraction was resuspended in a volume of SDS sample buffer equivalent to the volume of the soluble fraction, and aliquots of both fractions were analyzed by Western analysis. Soluble protein concentrations were determined by Bradford analysis (1976), and samples were precipitated (Bensadoun and Weinstein, 1976) before separation on SDS−PAGE slab gels (Laemmli, 1970). Proteins were transferred onto nitrocellulose using a Tyler Research Instruments semidry apparatus (Khyse-Anderson, 1984). After blocking, blots were incubated with a polyclonal anti-FTCD antibody, and detection was performed with [^{125}I]protein A as previously described (Murley *et al.*, 1993).

Purification of Histidine-Tagged Proteins. Wild-type FTCD was purified as described previously (Murley *et al.*, 1993). Histidine-tagged proteins were purified from frozen cell pellets as outlined below. Seventeen grams of BL21/DE3 cells expressing $FTCDH_6$ was thawed on ice in 3 vol of sonication buffer II (sonication buffer I with pH increased to 7.8 and 2-mercaptoethanol decreased to 10 mM) and lysed by sonication on ice for 3 min (12 × 15 s). After centrifugation (25000*g* for 30 min), the composition of the supernatant was altered to include the following: 0.1 M potassium phosphate (pH 7.8), 0.5 M NaCl, 5 mM histidine, 5 mM glutamate, 0.1% Triton X-100, 20% glycerol, and 10 mM 2-mercaptoethanol. This is also the composition of the binding buffer. The supernatant (80 mL) was added to 20 mL of a 50% slurry of Ni-NTA resin in Binding buffer and mixed end over end for 1 h at 4 °C. This mixture was packed into a column under gravity. The colum was washed with 3 column volumes of binding buffer and wash buffer (binding buffer with histidine increased to 50 mM). The protein was eluted at a rate of 15 mL/h using a linear gradient of 30 mL of wash buffer (pH 7.8) and 30 mL of 380 mM histidine in binding buffer (pH 7.3). Fractions containing activity were pooled and dialyzed overnight against 2 × 2 L of buffer B [25 mM MOPS (pH 7.3), 5 mM potassium phosphate (pH 7.3), 30% glycerol, 0.02% Triton X-100, 35 mM 2-mercap-

toethanol, and 1 mM benzamidine]. The dialyzed protein was further purified by Affigel 15-polyglutamate chromatography as reported for the purification of FTCD (Murley *et al.*, 1993). CDH_6 and $CD339H_6$ were purified as described for $FTCDH_6$.

As FTH_6 does not bind as tightly to the Ni-NTA resin, the Ni-NTA column was washed with 3 column volumes of binding buffer before eluting with a linear gradient of 5 to 150 mM histidine in binding buffer at pH 7.8. Fractions containing transferase active protein were pooled and dialyzed as above. The dialyzed protein was loaded onto a DEAE-Sepharose column equilibrated in buffer A (buffer B with glycerol decreased to 20%) and washed with 3 column volumes of the same buffer. FTH_6 was eluted with a linear gradient of 0 to 0.3 M KCl in buffer A.

N-Terminal Sequencing of the Deaminase Domain. Purified CDH_6 was bound to Ni-NTA resin and washed with 10 vol of 0.1 M potassium phosphate, pH 7.8, 0.5 M NaCl, 20% glycerol, 10 mM 2-mercaptoethanol, and 20 mM imidazole, to remove traces of Triton X-100. The enzyme was eluted using the same buffer with imidazole increased to 0.3 M. The protein was extensively dialyzed against H_2O to remove glycerol, prior to lyophilization. $CD339H_6$ was treated in the same manner and then further purified by chromatography on a reverse-phase C18 column (Vydac), using a gradient of 0 to 70% acetonitrile in 0.1% TFA, on a Hewlett Packard 1090M HPLC equipped with a diode array detector. The N-terminal amino acid sequences of CDH_6 and $CD339H_6$ were determined at the Biotechnology Research Institute, National Research Council of Canada (Montreal, Quebec), using an Applied Biosystems 470-A gas-phase sequencer coupled to a 120-A PTH-amino acid separation system.

Tryptic Digestion and Mass Spectral Analysis of the Transferase Domain. Purified FTH_6 was bound to Ni-NTA resin, extensively washed with 0.1 M potassium phosphate, pH 7.8, 0.5 M NaCl, and 10 mM imidazole to remove glycerol and detergent, and then eluted using the same buffer with imidazole increased to 0.3 M. Eluted FTH_6 was dialyzed against 2 × 1 L of 50 mM ammonium bicarbonate. Dialyzed FTH_6 (24 μg) was digested with 0.48 μg of TPCK-treated trypsin (Worthington Enzymes) in 50 mM ammonium bicarbonate (total volume of 85 μL) for 4 h at 37 °C. LC/MS analysis of 20 μL of the tryptic digest was performed by C. Fenwick and G. Tsaprailis, Concordia University. The peptides were separated using a Hewlett Packard 1090 HPLC with a reverse-phase C18 column (Vydac) and a gradient of 0 to 80% acetonitrile in 0.05% TFA, at a flow rate of 40 μL/min. The HPLC was directly coupled to a Finnigan-MAT SSQ 7000 mass spectrophotometer equipped with an electrospray ionization source.

Enzyme Assays and Channeling Experiments. Routine assays of transferase and deaminase activity were performed as previously described (MacKenzie, 1980; Drury & MacKenzie, 1975). The K_m value for formiminoglutamate (FIGLU) was determined at 30 °C in an assay mix containing 0.1 M potassium phosphate (pH 7.3), 35 mM 2-mercaptoethanol, 1 mM H_4PteGlu, and various concentrations of FIGLU. The H_4PteGlu K_m value was determined using 5 mM FIGLU and various concentrations of (6*R*,*S*)-H_4PteGlu. To determine the $H_4PteGlu_5$ K_m value, the assay mix included 5 mM FIGLU and various concentrations of (6*R*,*S*)-$H_4PteGlu_5$, with 118 mM NaCl. The kinetic constants for the deaminase activity were determined at 30 °C using the assay conditions described by Paquin *et al.* (1985), with 2-mercaptoethanol decreased to 35 mM. The data from these experiments were fit to the Michaelis−Menten equation using the nonlinear regression analysis program Enzfitter (Leatherbarrow, 1987).

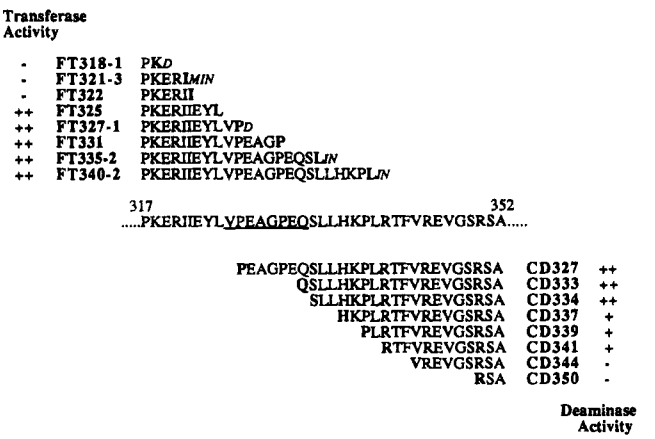


FIGURE 1: Analysis of the interdomain region of FTCD. The sites of termination of translation of the N-terminal FT domains and initiation of translation of the C-terminal CD domains are indicated in bold (initiator methionines are not shown). Nonrelated residues attached to the N-terminal domains are written in italics. The minimum linker region of 8 residues is underlined. The level of enzyme activity in *E. coli* extracts is indicated: +, >0.001 and <0.1 μmoles/min/mg; ++, >0.1 μmoles/min/mg; −, not detectable.

Gel Filtration. Purified samples of FTCD, $FTCDH_6$, FTH_6, and CDH_6 were analyzed on a Superose 6 HR 10/30 column equilibrated in 0.1 M potassium phosphate (pH 7.3), 20% glycerol, 0.02% Triton X-100, and 35 mM 2-mercaptoethanol. Aliquots containing 200 μL of approximately 2 mg/mL protein were injected onto the column and chromatographed at a flow rate of 0.2 mL/min. Absorbance of the eluate was monitored at OD_{280}. Ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen (25 kDa) were used as molecular mass standards. The void volume was determined using blue dextran.

RESULTS

Deletion Analysis of FTCD. The plasmids expressing N- and C-terminal fragments of FTCD contained the same translational enhancer sequence, ribosome binding site, and intervening sequence up to and including the initiation codon. The plasmids were transformed into BL21/DE3 cells, and the resulting protein products were analyzed by enzyme assay and Western blotting of cell extracts. Figures 1, 2, and 3 summarize the results from these analyses.

The FT mutants (Figure 1) begin at the same initiator methionine as the wild-type FTCD and end between residues 318 and 335. As shown in Figure 2A, mutants ending at or before residue 322 are completely insoluble, while larger, transferase-active fragments are at least partially soluble. FT325 partitions between the soluble and insoluble fractions.

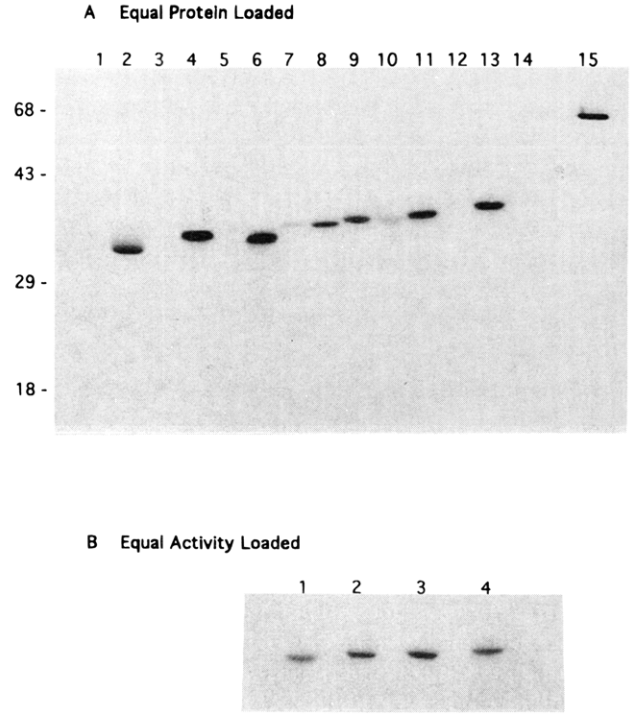


FIGURE 2: Western analysis of the N-terminal fragments. (A) Lanes 1−14 contain insoluble and soluble fractions of *E. coli* extracts expressing the following N-terminal fragments: (1 and 2) FT318-1, (3 and 4) FT321-3, (5 and 6) FT322, (7 and 8) FT325, (9 and 10) FT327-1; (11 and 12) FT331, (13 and 14) FT335-2. Odd-numbered lanes contain 25 μg (1, 3, and 5) or 5 μg (7, 9, 11, and 13) of soluble protein. Even-numbered lanes contain volume equivalent amounts of insoluble fractions. Lane 15, 10 ng of purified FTCD. (B) Lanes with aliquots of soluble extract containing 5 nmol/min of transferase activity: (1) FT325, (2) FT327-1, (3) FT331, and (4) FT335-2.

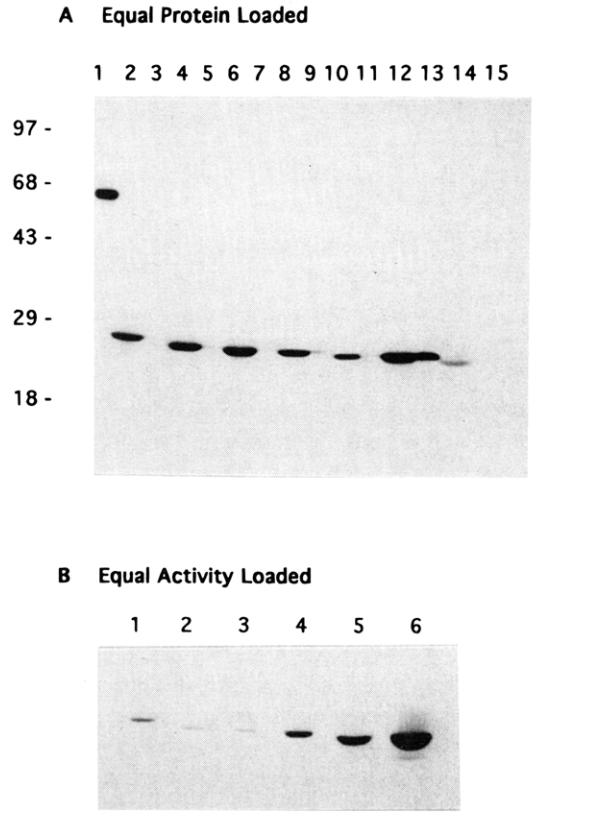


FIGURE 3: Western analysis of the C-terminal fragments. (A) Lane 1, 10 ng of purified FTCD. Lanes 2−15 contain insoluble and soluble fractions of *E. coli* extracts expressing the following C-terminal fragments; (2 and 3) CD327, (4 and 5) CD333, (6 and 7) CD334, (8 and 9) CD337, (10 and 11) CD339, (12 and 13) CD341, and (14 and 15) CD344. Even-numbered lanes contain 5 μg of soluble protein. Odd-numbered lanes contain volume equivalent amounts of insoluble fraction. (B) Lanes with aliquots of soluble extract containing 1 nmol/min deaminase activity: (1) CD327, (2) CD333, (3) CD334, (4) CD337, (5) CD339, and (6) CD341.

When aliquots of soluble extract containing equivalent units of transferase activity were analyzed by Western blotting (Figure 2B), each lane contained approximately equal amounts of immunoreactive protein. This indicates that FT325, FT327-1, FT331, and FT335-2 have similar intrinsic transferase activity.

To isolate a C-terminal fragment which did not contain sequence overlap with the smallest transferase active domain, CD327 was constructed. As shown in Figure 1, CD327 expresses cyclodeaminase activity. To delineate the N-terminal boundary of the deaminase active domain, mutants beginning at positions further 3′ were constructed, and the expressed proteins were analyzed in terms of their solubility and deaminase activity. Truncated proteins initiating at or before residue 341 expressed some level of deaminase activity, while smaller fragments were completely inactive. All constructs produced soluble protein (Figure 3A). However, CD341 was susceptible to proteolysis, and CD344 and CD350 were not as highly expressed as their larger counterparts. When aliquots containing equivalent units of deaminase activity are compared (Figure 3B), it is clear that the intrinsic deaminase activity is highest for CD333 and CD334 and decreases dramatically as the domain is shortened.

Expression and Purification of the Histidine-Tagged FTCD and Domains. Because the isolated domains were very difficult to purify by conventional methods, hexahistidine tags were appended to the C-terminal ends of the full-length FTCD ($FTCDH_6$), the transferase active domain (FTH_6), the deaminase active domain (CDH_6), and a shorter deaminase active fragment, CD339 ($CD339H_6$). Ni-NTA and polyglutamate affinity chromatography effected a 150-fold purification of $FTCDH_6$ and a 100-fold purification of CDH_6, while Ni-NTA followed by DEAE chromatography produced a 60-fold purification of FTH_6. SDS−PAGE of the purified histidine-tagged proteins is shown in Figure 4.

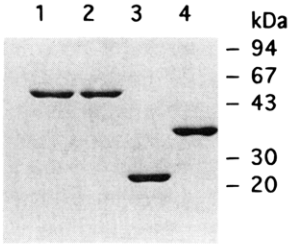


FIGURE 4: SDS−PAGE analysis of purified proteins. Proteins were electrophoresed on a 12% acrylamide gel and stained with Coomassie Brilliant Blue. Each lane contains 10 μg of purified product. (1) FTCD, (2) $FTCDH_6$, (3) CDH_6, and (4) FTH_6.

Sequencing of CDH_6 and $CD339H_6$ yielded the expected 10 amino-terminal residues and confirmed that these proteins initiated at amino acids 333 and 339, respectively. While CDH_6 retained the initiator methionine, the majority of $CD339H_6$ did not.

The combination of reverse-phase HPLC and ESI-MS was used to separate and identify tryptic fragments of the

Table 1: Comparison of Subunit and Native Molecular Masses

protein	mol mass (kDa) subunit[a]	native[b]
FTCD	59	438
$FTCDH_6$	60	380
FTH_6	37	83
CDH_6	24	52

[a] Subunit size taken from amino acid sequence. [b] Native size determined by gel filtration.

N-terminal domain FTH_6. Fragments covering greater than 90% of this domain were positively identified by mass spectroscopy. A fragment of 2040.7 Da was of particular interest since it corresponded exactly to the predicted mass of the C-terminal tryptic fragment (containing residues 321−328 plus the histidine tag).

Quaternary Structure of FTCD and Isolated Domains. Gel filtration was used to determine the association state of FTCD and the isolated domains. Both FTCD and $FTCDH_6$ eluted as high molecular weight complexes (Table 1), suggesting that they exist as the previously described octamer. FTH_6 and CDH_6 eluted at apparent molecular weights of twice their subunit sizes, indicating that both are dimers. Cross-linking of the full-length enzyme and of the isolated domains with bis[sulfosuccinimidyl]suberate (Pierce, Rockford, IL) supported these results (data not shown).

Kinetic Characterization of the Recombinant Enzymes. Table 2 contains kinetic parameters for the wild-type and histidine-tagged proteins. The transferase activities displayed K_m values for FIGLU in the millimolar range, as previously observed with enzyme isolated from pig liver (Beaudet & MacKenzie, 1975). K_m values for (6*S*,*R*)-H_4PteGlu are also similar between the transferase enzymes. However, the K_m value of FTH_6 for the pentaglutamylated substrate (6*S*)-$H_4PteGlu_5$, at greater than 75 μM, is over 50 times higher than that of $FTCDH_6$ under identical conditions. This observation is in agreement with the K_m value previously described for a transferase active dimer isolated by denaturation of the octamer (Findlay & MacKenzie, 1988).

The K_m values for (6*S*)-formimino-H_4PteGlu were similar for the three deaminase enzymes assayed, but approximately one-half to two-thirds the value described elsewhere (Paquin *et al.*, 1985; Findlay & MacKenzie, 1988). The K_m values for the pentaglutamate derivative were 1.4 μM for the deaminase activity of $FTCDH_6$ and 19 μM for CDH_6. CDH_6 and a deaminase active dimer produced by denaturation (Findlay & MacKenzie, 1988) display essentially identical K_m values for the pentaglutamylated substrate. Thus the deaminase domain retains significant polyglutamate specificity, while the transferase domain does not. The k_{cat} values for each isolated domain are approximately half that of the corresponding activity in FTCD.

Channeling of the Formimino Intermediate between Active Sites. Channeling experiments (Figure 5) were performed using 50 μM $H_4PteGlu_1$ or $H_4PteGlu_5$ and equivalent units of enzyme activity. When FTCD is used with the monoglutamylated substrate (Figure 5A), we observe that the rate of formation of methenyl-$H_4PteGlu_1$ is much slower than the rate of the transferase (formimino + methenyl), indicating that the formimino intermediate is accumulating in the medium. However, when $H_4PteGlu_5$ is used as substrate (Figure 5B), this intermediate is channeled to the deaminase active site and the rate of formation of the second product is similar to that of total products. The same phenomenon is observed using $FTCDH_6$, as shown in panels C and D, although the efficiency of channeling is decreased. When FTH_6 and CDH_6 are mixed, the results (panels E and F) indicate that the isolated domains cannot channel the pentaglutamylated intermediate. In contrast to $FTCD(H_6)$, an increase in transferase activity is not observed when the pentaglutamylated substrate is substituted for the monoglutamate with the mixed domains.

DISCUSSION

A Short Linker Sequence Separates the N-Terminal Transferase Domain and the C-Terminal Deaminase Domain. Most multifunctional enzymes comprise distinct modules or domains linked together by short flexible linker sequences (Coggins & Hardie, 1986). Previous studies including limited proteolysis of FTCD and denaturation of the octamer indicated that the transferase and deaminase activities might be located on different domains. As expression of functionally active fragments demonstrates the modular composition of a protein, we attempted to experimentally define the domain structure of FTCD through deletion mutagenesis.

As FTCD does not show significant homology to other known proteins, sequence similarity could not be used to estimate its domain structure. However, Findlay *et al.* (1989) had previously observed that both a 39-kDa transferase active proteolytic fragment and the full-length FTCD appear to be *N*-terminally blocked. We interpreted this to mean that a protease-sensitive sequence connecting the transferase and deaminase domains might lie within amino acids 320−360. Using deletion mutagenesis techniques, we produced constructs which would express N-terminal proteins ending at different positions between amino acids 318 and 340 and C-terminal fragments which initiated at different residues within the same region.

The N-terminal boundary of the linker sequence resides within residues 324−326. Proteins ending at or before residue 322 apparently do not fold readily and are completely insoluble, while larger proteins ending at or after residue 325 are soluble and equally active. Although all C-terminal peptides were at least partially soluble, CD327, CD333, and CD334 have substantially higher turnover numbers than CD337, CD339, and CD341. The smallest C-terminal fragments, CD344 and CD350, are completely inactive. Therefore, the C-terminal boundary of the linker region lies within residues 333−335 and distinguishes the fully active CD fragments from those which are less active.

Thus each FTCD subunit includes an N-terminal transferase domain and a C-terminal deaminase active domain, separated by a short linker. This linker sequence is between 8 (as underlined in Figure 1) and 12 residues long and is similar in composition to other linkers described by Argos (1990). We can remove 6 of these proposed linker residues (327 to 332, inclusive) to produce a protein which is less soluble than the wild-type FTCD yet still expresses both activities, albeit at reduced levels. Upon extending this deletion to include residues 327−338, the protein becomes even less soluble and can no longer catalyze either reaction (data not shown).

For the remainder of these studies we chose to produce and purify the full-length enzyme and the isolated domains

Table 2: Kinetic Properties of the Transferase–Deaminase Enzymes

enzyme	K_m FIGLU (mM)	K_m (6*R*,*S*)-H_4PteGlu (μM)	K_m (6*S*)-H_4PteGlu$_5$ (μM)	K_m (6*S*)-formimino H_4PteGlu (μM)	K_m (6*S*)-formimino H_4PteGlu$_5$ (μM)	k_{cat} (s^{-1}) transferase	k_{cat} (s^{-1}) deaminase
FTCD	5.8 ± 0.3	141 ± 7		66 ± 7		58 ± 3	394 ± 2
$FTCDH_6$	6.7 ± 1.1	148 ± 13	1.7 ± 0.4	70 ± 4	1.4 ± 1.3	77 ± 7	379 ± 25
FTH_6	8.3 ± 0.9	111 ± 5	>75			32 ± 1	
CDH_6				70 ± 5	19 ± 2		213 ± 8

[a] Values are expressed as averages ± SD for 3–6 separate determinations.

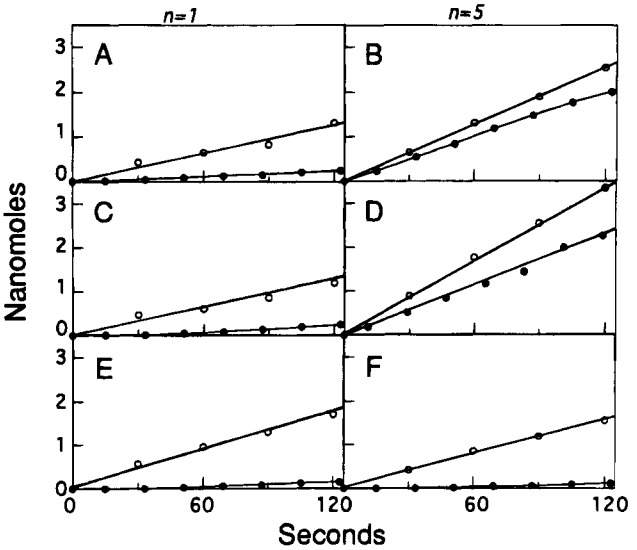


FIGURE 5: Time course of appearance of products. The products represented are (○) transferase activity, the sum of 5-formimino-H_4PteGlu$_n$ and 5,10-methenyl-H_4PteGlu$_n$, and (●) deaminase activity, 5,10-methenyl-H_4PteGlu$_n$ alone. (A and B) FTCD, (C and D) $FTCDH_6$, and (E and F) a matched mixture of FTH_6 and CDH_6.

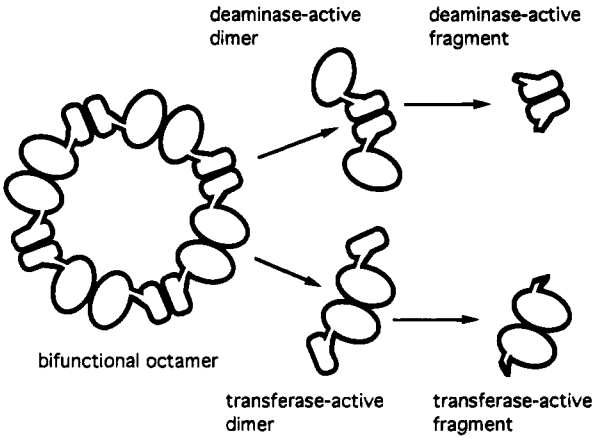
CD333 and FT328 as histidine-tagged fusion proteins to simplify their purification.

Octameric FTCD Contains Two Types of Subunit Interfaces. Previous work on FTCD suggested that formation of the octamer is required for co-expression of both catalytic activities and for channeling of substrate between active sites. In the proposed model the eight subunits are arranged to form a circular tetramer of dimers, with two types of subunit interfaces (MacKenzie et al., 1980; Findlay & MacKenzie, 1987, 1988). Therefore, each domain might include sequences governing subunit dimerization. Analytical gel filtration indicated that both isolated domains exist as dimers, confirming that octameric FTCD includes two different types of subunit interfaces as represented by the tetramer of dimers shown in Scheme 1.

Indirect evidence suggests that both the transferase and deaminase activities are dependent on the retention of specific subunit interfaces (Findlay & MacKenzie, 1987, 1988). If it is a prerequisite for either activity, dimerization may be required to achieve an active conformation of the subunit, or to form one or more catalytic sites at a subunit interface using residues derived from each monomer. Determining the number and position of catalytic sites within the octamer is required to resolve these questions.

As both domains reside within one subunit, it seemed reasonable that the isolated domains might continue to associate noncovalently. Several attempts were made to demonstrate such an interaction, without success. The

Scheme 1



domains could not be cross-linked using a bifunctional reagent, and neither domain could be retarded on a Ni-NTA column previously loaded with the H_6 version of the other domain. As well, the fluorescence spectrum of an equimolar mixture of CDH_6 and FTH_6, initially incubated at high protein concentration (1 mg/mL), is identical to the spectrum obtained by mathematically adding the individual spectra of each domain. Finally, lack of channeling by domains incubated at high protein concentration before assay indicates the absence of a specific heterodomain interaction in this system. The apparent lack of association between the isolated domains emphasizes the importance of the linker region in maintaining domain interactions within each subunit of the octamer.

The isolated domains retain kinetic characteristics similar to that of the native enzyme with monoglutamate substrates. The major difference is a decrease in their k_{cat} values to about 50%. Previously, a chymotryptic transferase active dimeric fragment was also shown to have only 67% of the expected activity (MacKenzie *et al.*, 1980). While the domains retain a great deal of integrity, it is likely that their removal from the restraints imposed by the octameric structure results in some conformational changes.

Role of the Polyglutamate Tail in Substrate Binding and Channeling. Like many other folate-dependent enzymes, FTCD displays a preference for polyglutamylated substrates. Polyglutamylation improves the binding of both the folate substrates and FIGLU (Paquin *et al.*, 1985; Findlay *et al.*, 1989). As well, a polyglutamate tail, four or more polyglutamates in length, is required for the direct transfer of formimino-H_4PteGlu$_n$ between active sites. Paquin *et al.* (1985) demonstrated the presence of only four high-affinity polyglutamate binding sites per octamer, or one per dimer. Our results indicate that this polyglutamate binding site resides within the deaminase domain. The existence of only

one polyglutamate binding site per pair of deaminase domains suggests that it (and possibly the deaminase active site) may be located at the subunit interface formed between deaminase domains.

Basic amino acids, in particular arginine residues, have been implicated in the binding of polyglutamylated substrates to other folate-dependent enzymes (Kamb *et al.*, 1992; Maras *et al.*, 1994; Finer-Moore *et al.*, 1994). Rabinowitz's group suggested that at 53-residue sequence, which is specific to 10-formyltetrahydrofolate synthetases that bind longer polyglutamates and includes doublets of basic amino acids, may constitute part of that enzyme's polyglutamate binding site (Whitehead & Rabinowitz, 1988; Nour & Rabinowitz, 1992). Within FTCD, arginine doublets (R381,R382; R392,R393; R435,R436) are only located in the deaminase domain; some of these doublets may aid the binding of polyglutamate to the octamer.

Since pentaglutamylated substrate is transferred with the highest efficiency, while longer polyglutamates are more tightly bound (Paquin *et al.*, 1985), substrate channeling within FTCD is thought to involve a steric component. MacKenzie and Baugh suggested a "swinging arm" mechanism whereby the polyglutamate binds to a site on the octamer while the more mobile pterin moiety can interact with either type of catalytic site (1980). As we have shown that the activities reside in different domains, the substrate must be channeled between domains, if not subunits.

As predicted by this model, the separated domains are not able to channel pentaglutamylated intermediate. While some method of direct transfer between sites within the octamer is likely, the slightly altered properties of the CDH_6 domain prevent us from entirely ruling out a "release and rebinding" mechanism. Similar limitations were encountered in testing this model by chemical modification of the native proteins (MacKenzie & Baugh, 1980).

A swinging arm is not the only feasible method of direct transfer. Recently Knighton *et al.* (1994) described a possible mechanism for channeling of polyglutamylated dihydrofolate within bifunctional thymidylate synthase–dihydrofolate reductase of *Leishmania major*. They observed an unusual distribution of charged residues across the surface of the protein which may serve to guide the intermediate between sites. A similar "electrostatic highway" (Stroud, 1994), the proposed model of a polyglutamate "anchor" or a third unknown mechanism may mediate the channeling described for FTCD. Crystallographic analyses of FTCD and its isolated domains should clarify this issue and, as well, answer questions regarding the number and location of active sites in relation to the subunit interfaces formed by each domain.

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