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The objective of this project is to test the hypothesis that the complex proteins with novel functions arose in the course of evolution by combining structural domains having partial functions. The research focuses on the enzymes that catalyze *de novo* pyrimidine biosynthesis. While the reactions are the same in most organisms, there are striking differences in the structure and regulation of these enzymes. This year we completed the sequence of the mammalian protein CAD, a 243 kDa polypeptide which carries gl ine dependent (GLN) carbamyl phosphate synthetase (CPS), aspartate transcarbamylase (AT d dihydroorotase (DHO) activities. Phylogenetic analysis suggests that the mammalian chimeric pro s formed by stepwise translocation and fusion of ancestral genes that occurred prior to the major radiation that lead to fungi, plants and animals. The sequence divergence suggests that the fused and monofunctional DHO domains have a different evolutionary history. In contrast, sequence studies and molecular modeling of the mammalian ATC domain and a reexamination of the subunit structure of a class A ATCase, thought to have a radically different structure, showed that the ATCase catalytic domain is a trimer of identical 34 kDa polypeptides that has been highly conserved throughout the course of evolution.

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FINAL REPORT ON CONTRACT N00014-87-K-0081 R&T CODE 4413031

PRINCIPAL INVESTIGATOR: David R. Evans

CONTRACTOR: Wayne State University, Detroit, Michigan

CONTRACT TITLE: The Evolution and Analysis of the Functional Domains of the Chimeric Proteins that Initiate Pyrimidine Biosynthesis.

PROJECT PERIOD: December 1, 1987 - July 31, 1990

RESEARCH OBJECTIVE: To determine the structural organization and trace the evolutionary development of the complex multi-domain proteins involved in *de novo* pyrimidine biosynthesis. The enzymes which catalyze the first three steps in the pathway, glutamine dependent (GLN) carbamyl phosphate synthetase (CPS), aspartate transcarbamylase (ATC) and dihydroorotase (DHO) are separate proteins in eubacteria, but are consolidated in a single 243 kDa chimeric polypeptide in mammals and other higher eukaryotes.

PROGRESS REPORT

We have looked at various aspects of the evolution of the multidomain protein and conclude:

1. The trifunctional mammalian polypeptide probably arose through a complex series of gene duplications, fusions and extinctions.

We have sequenced two overlapping cDNA clones which together span the entire 6.5 kb CAD coding region. While the GLN, CPS and ATC domains are clearly homologous (approximately 50% sequence identify) to the corresponding monofunctional *E. coli* enzymes, the mammalian DHO domain is strikingly different than its bacterial counterpart. Another surprising discovery, was that the mammalian DHO domain is homologous to the long interdomain linker region that connects the CPS and ATC domains in yeast but very different than the functional yeast DHOase which is encoded by a separate gene. The phylogenetic analysis shows that the monofunctional and fused DHOases have a different evolutionary history since the dendrogram does not conform to the accepted phylogeny of the organisms represented. Partial sequence data for the pyrimidine biosynthetic complex of *Dictyostelium discoideum* (Faure et al., Eur. J. Biochem. 179: 345, 1989) clearly demonstrated that the fusion of constituent polypeptides occurred much earlier in the course of evolution than heretofore expected. Although the *Dictyostelium* lineage predates the major radiation that lead to fungi, plants and animals, the dendrogram clusters the enzyme from *Dictyostelium* with higher eukaryotes and the yeast enzymes with the prokaryotic DHOases.

Our current interpretation is that the disparate sequences of the two classes of DHOases can be plausibly explained by divergent evolution following duplication of an ancestral gene. According to this model, the fusion of CPSase and ATCase genes, separated by an approximately 300 bp spacer, occurred sometime between the divergence of bacteria

and slime molds. This early event was followed by duplication of a monofunctional DHOase gene, one copy of which was translocated and inserted into the spacer region. Perhaps initially non-functional, reactivation in the *Dictyostelium* and metazoan lineages, with the concomitant advantages of coordinate regulation, lead to the extinction of the monofunctional dihydroorotase. Reactivation did not occur in yeast and the separate monofunctional DHOase was preserved. If this explanation is correct all of the dihydroorotases are descendants of a common ancestor and the sequence differences between the two families is a consequence of differences in structural constraints imposed on the fused and monofunctional dihydroorotases.

2. Aspartate Transcarbamylases from all known species consist of trimeric catalytic subunits composed of identical 34 kDa polypeptides.

The hypothesis that multifunctional proteins arose by consolidation of genes encoding monofunctional enzymes and that the architecture of the domain is very ancient suggests that the enzymes from all organisms should have homologous structures. However prokaryotic ATCases have been reported to be highly polymorphic. Class B and class C ATCases have trimeric catalytic subunits consisting of 34 kDa polypeptides. The major difference is that the class B enzymes have regulatory subunits while the class C enzymes are unregulated. In contrast the class A enzymes, the largest ATCase molecules, were reported to be dimers of 180 kDa polypeptides. The extraordinary size of the polypeptide is large enough to include domains catalyzing other steps in the pathway.

We have re-examined the subunit structure of *P. fluorescens* ATCase, the best characterized class A enzyme. The molecular weights of CPS and DHO are 160 kDa and 86 kDa respectively, comparable to the size of the *E. coli* enzymes, while the ATCase is much larger. The material we have isolated represents the purest active enzyme preparation thus far obtained. Contrary to previous reports we found that the molecule consists of two subunits, 34 kDa and 45 kDa, which are present in approximately stoichiometric amounts in the complex. The catalytic activity is associated with the 34 kDa polypeptide and while the function of the 45 kDa polypeptide is not known, it may be involved in regulation. Sedimentation velocity and gel filtration studies showed that the enzyme is a stable complex with a molecular weight of approximately 480,000 and a Stokes radius of 77 Å.

Thus the enzyme is a dodecamer composed of six copies of each of the two types of subunit. The structural organization is similar to that of the *E. coli* enzyme although the putative regulatory subunits are much larger. All ATCase molecules characterized thus far have catalytic subunits that are trimers of 34 kDa polypeptides supporting the hypothesis that they all evolved from a highly conserved ancestral domain.

3. The tertiary structure of the catalytic domain of mammalian and bacterial aspartate transcarbamylase is highly conserved.

The sequence and structure of the fused and monofunctional aspartate transcarbamylase domains are highly homologous. We built an energy minimized model of the mammalian ATCase domain using the x-ray coordinates of the *E. coli* enzyme as a tertiary template Favorable hydrophobic interactions, a compact globular shape and a normal distribution of hydrophobic and hydrophillic side chains suggests that the model is a plausible representation of the structure of the mammalian ATCase domain. The backbone carbonyl carbons are nearly superimposable, the active site regions are virtually identical and interactions between the subdomains in the monomer were conserved. However there was extensive remodeling of the trimeric contacts. These studies suggested that while the fundamental architecture of the aspartate transcarbamylase domain is very ancient, there

may have been adaptive changes which reflect the different structural context and regulatory mechanisms of the bacterial and mammalian proteins.

4. The kinetic parameters of aspartate transcarbamylases are adapted to the functional constraints dictated by their environment.

In the latter part of the contract period we focused our efforts on the aspartate transcarbamylase from six cryophillic, mesophillic and thermophillic strains of *Bacillus*. We measured the thermostability of aspartate transcarbamylase in cell extracts and found a wide range of melting temperatures (66 - 83) which approximately correlated with the growth optimum of the mesophillic and thermophillic organisms. The only cryophile studied, *B. insolitus*, had a denaturation temperature similar to that of the mesophillic organisms.

Steady state kinetic studies have shown that while the mesophillic *Bacillus* species have aspartate transcarbamylase kinetic parameters similar to the *E. coli* and mammalian enzymes, the K_m for the thermophillic proteins is 10-20 fold lower indicating much stronger aspartate binding. Enhanced aspartate binding could result from more or stronger interactions between the enzyme and substrate but the most interesting possibility is that it is a consequence of stronger inter-domain interactions. In the *E. coli* enzyme aspartate binding results in closure of the two domains which completes the formation of the active site. Moreover, when the assays of some of the thermophillic proteins were conducted at elevated temperatures, the K_m increased. Clearly there have been adaptive changes which allow the molecule to function efficiently at elevated temperatures.

INVENTIONS

None.

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TRAINING ACTIVITIES:

A total of 2 graduate students, 1 undergraduate and 1 postdoctoral were supported by this contract. This included 2 women and 2 minority.