On the Origins of Esterases[1]

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Comparisons among the primary sequences of five cloned eukaryotic esterases reveal two distinct lineages, neither bearing any significant overall sequence similarity to the functionally related serine protease multigene family. We have not eliminated the possibility that the esterases may have residual conformational similarities to the serine proteases. However, our profile analysis and analyses of the predicted conformations of the esterases reveal little similarity to the serine proteases. Four of the esterase proteins share 27%–53% overall sequence similarity and evidence of a catalytic mechanism involving the same Arg-Asp-Ser or His-Asp-Ser charge relay. We propose that these four esterases, three of them cholinesterases, form part of a multigene family essentially separate from the serine proteases.

Introduction

The origin of multigene families by gene duplication and subsequent divergence is a cornerstone of the theory of molecular evolution in higher eukaryotes (Ohno 1970). A widely accepted corollary is that functional similarity among different proteins often reflects genetic relatedness. It is therefore important to note contrary evidence from comparisons of the sequences of five cloned esterase genes postulated to belong to a multigene family of serine hydrolases.

All higher eukaryotes have many distinct esterases, several of which are classified as nonspecific carboxylesterases on the basis of their broad substrate specificities. These carboxylesterases have been subdivided into A, B, and C types on the basis of differential patterns of inhibition by organophosphates (Heymann 1980). Among the B-type carboxylesterases are the cholinesterases, which are further subdivided into acetyl and butyryl cholinesterases on the basis of their preferred substrates (Silver 1974, p. 413).

There are several lines of evidence that support the hypothesis proposed by Augustinsson (1968) that the esterases are phylogenetically related to the serine proteases and thus comprise a serine hydrolase multigene family. Some characteristics of the esterases and proteases that support this hypothesis are summarized in table 1. Perhaps the strongest evidence comes from analyses of tryptic peptides containing part of the catalytic (esteratic) site that is isolated from several carboxylesterases by its binding to the substrate analogue diisopropyl fluorophosphate (DFP). The sequences of these peptides share a consensus octapeptide containing a Ser residue directly involved in the hydrolytic reaction (Dayhoff et al. 1972). The catalytic sites of several proteases have also been isolated and characterized by these means and likewise contain a con-

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Table 1
Similarities between Carboxylesterases and Serine Proteases

Characteristic	Reference(s)
1. Three-in-eight match between consensus active-site sequences including invariant serine	Dayhoff et al. 1972
2. DFP inactivates enzymes by binding irreversibly to the active-site serine	Krisch 1971
3. Inhibition by organophosphates and carbamates	Augusteyn et al. 1969
4. Overlapping substrate specificity	Heymann 1980, Previero et al. 1983

sensus octapeptide including an invariant Ser (Young et al. 1978). Moreover, there is a three-in-eight match between the two consensus sequences, suggesting their origin from a common ancestor (Neurath 1984).

Evolutionary Relationships among The Serine Hydrolases

Subsequent cloning and sequencing of several serine proteases has directly confirmed that they are phylogenetically related to one another (Rogers 1985). Indirect evidence indicating relationships among the esterases derives from the findings of clusters of tightly linked carboxylesterase genes in rodents and insects (Zouros et al. 1982; Hedrich and von Deimling 1987) and of allelic differences in substrate specificity and inhibition characteristics of esterases in *Drosophila* (Pen et al. 1986). Similarly, indirect support for the postulated relationship between the esterases and serine proteases derives from the observation that some proteases have esterase activity and that many esterases also have protease activity (Heymann 1980). There is also indirect evidence that cholinesterases have a catalytic mechanism similar to that known for the serine proteases (Silver 1974, p. 26; Rosenberry 1975).

However, contrary conclusions must now be drawn from full sequence comparisons among two acetylcholinesterases (AChE) from the *Torpedo californica* ray (Schumacher et al. 1986) and *Drosophila melanogaster* (Hall and Spierer 1986), a butyryl cholinesterase (BuChE, also termed serum cholinesterase) from man (Lockridge et al. 1987), and two B-type carboxylesterases, EST D from man (Lee and Lee 1986) and EST 6 from *D. melanogaster* (Oakeshott et al. 1987). The overall sequence similarities of the *Torpedo* and *Drosophila* AChE proteins are 38% to each other; 53% and 39%, respectively, to the BuChE; and 30% and 27%, respectively, to EST 6. BuChE and EST 6 also show 30% similarity to one another. All these similarity values are highly significant (minimum of 19 SDs above expectations assuming no similarity; see Doolittle 1981). On the other hand, EST D shows no significant sequence similarity to any of the other four esterases (maximum of 0.5 SD above expectation).

We have used profile analysis (Gribskov et al. 1987) to determine whether any of the four related esterases are similar in sequence to any of the 4,753 protein sequences in the National Biomedical Research Foundation protein data base (Release 12.0). The only protein to show significant sequence similarity to a probe derived from the four related esterases is bovine thyroglobulin, which was previously recognized as sharing substantial sequence similarity with *Torpedo* AChE (Schumacher et al. 1986). None of the serine proteases in the data base shows significant similarity to the esterase probe. Clearly these esterases at least cannot be accommodated with the serine proteases in a serine hydrolase multigene family. Furthermore, EST D appears to represent a lineage separate from that of the other four esterases.

Table 2
Regions Surrounding Residues (in Boldface) Proposed to Participate in the Charge-Relay System of the Catalytic Site of Some Representative Serine Proteases

	His Region		Asp Region		Ser Region
	37		80		170
Sg Tryp . . .	LyAqdiVLTAA**H**CVsGsG	. . (42aa) . .	gyNgtgK**D**wALI	. . (89aa) . .	GD**S**GGPMf
	39		82		173
H Tryp . . .	sISkryVLTAA**H**CLvGKS	. . (42aa) . .	NSirLiN**D**IGLI	. . (91aa) . .	GD**S**GGPLV
	71		116		210
D Tryp . . .	IYSaNiIVTAA**H**CLqSvS	. . (44aa) . .	NaNTMvN**D**IAVI	. . (93aa) . .	GD**S**GGPLV
	46		90		183
B Tryp . . .	LINsQwVVSAA**H**CykSgi	. . (43aa) . .	NSNTLNN**D**ImLI	. . (92aa) . .	GD**S**GGPVV
	57		102		195
B Chyt . . .	LiNeNwVVTAA**H**CgvTtS	. . (43aa) . .	NSlTINN**D**ITLL	. . (92aa) . .	GD**S**GGPLV

NOTE.—Sequences were aligned using the ALIGN program of the National Biomedical Research Foundation and the mutation data matrix (Dayhoff et al. 1983). The position of each catalytic site residue within the sequence is shown above each sequence. Residue numbering begins with 1 as the first amino acid of the mature protein. Uppercase letters indicate identical amino acids or conservative substitutions compared with the consensus residue; lowercase letters indicate nonconservative substitutions. Amino acid pairs having log-odds scores ⩾ +1 were considered to be conservative substitutions (Dayhoff et al. 1983). The number of amino acid residues between the residues in boldface is given in parentheses. Sg, H, D, and B Tryp are trypsins from *Streptomyces griseus* (Young et al. 1978), oriental hornet (Janey et al. 1983), *Drosophila* (Davis et al. 1983), and cattle (Titani et al. 1975), respectively. B Chyt is chymotrypsin A of cattle (Blow et al. 1969).

Catalytic Residues of Esterases

How then to explain the evolution of similar functions in such separate lineages? Definitive answers require full knowledge of the catalytic mechanism and tertiary structure of a representative of each lineage, and such data are only available for the serine proteases. However, much can be inferred, given these baseline data for the serine proteases, from the primary sequences of the five esterases and some knowledge of the catalytic mechanisms of the cholinesterases (Rosenberry 1975).

Intensive analyses of several serine proteases have shown that the catalytic site of each comprises three key residues involved in a charge relay to donate protons to—and so to hydrolyze—a peptide bond (Price and Stevens 1982, pp. 172–175; Craik et al. 1987). These three residues—the reactive Ser cited above, the basic His, and the acidic Asp—are not contiguous in the primary structure but are each embedded in highly conserved regions of 8–18 residues. The relative positions of these three regions in the primary sequence are also highly conserved, with approximately 43 residues between the His and Asp and then approximately a further 91 residues to the Ser (table 2; Young et al. 1978).

As explained above, EST 6 and the three cholinesterases sequenced also contain a consensus octapeptide including an invariant Ser. Figure 1 shows an alignment of these four esterases in which the invariant Ser residue appears at position 277. An invariant Asp, which is also embedded in a highly conserved region, is found at position 249. The high degree of conservation around these residues suggests a critical role in catalytic function.

On the basis of analogy to the His-Asp-Ser charge-relay system of the serine proteases, one would predict a conserved His residue on the amino-terminal side of the conserved Asp. No such His exists in these molecules; however, a conserved Arg residue that is embedded in a region of conserved amino acids is found at position 220. Indirect evidence to support the involvement of this Arg in the charge relay comes from work on the phosphodiesterase, RNase ST, in which, in contrast with RNase, an Arg residue is utilized in place of a His in the active site (Nakamura et al. 1982).

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             1                                                            60
Torpedo AChE ..........  ......Mnll  tvssLgvlLh  LvvlcqaDdh  sELlVnTKSG  KVmGtrVpVL
Human BuChE  ..........  .mhSkvtllc  lrFlFwFVLL  cMLlGkShTE  DDIllaTKnG  KVRGmnlTVF
Dros. AChE   malsrqsrv   lpmSlpLplt  lpLpLVLVLs  LhLSGvcgvl  DrLvVQTsSG  pVRGRsVTVq
Dros. EST 6  ..........  .....mnyvg  lgLllVLscL  wLgSnaSDTD  DpLlVQlpqG  KlRGR....d
Consensus    ----------  ---S------  I-L-LVLVLL  L-LSG-SDT-  D-L-VQTKSG  KVRGR-VTV-

             61                                                          120
Torpedo AChE sshlsAFLGI  PFAEPPVGnM  RFRrPEPkK.  pWSgVWnAst  YPNnCqQyvD  eqFPGFSGSE
Human BuChE  GGtVtAFLGI  PYAqPPlGrL  RFkKPqslt.  kWSdlWnATK  YaNsCcQnlD  qsFPGFhGSE
Dros. AChE   GreVhvYtGI  PYAkPPVeDL  RFRKPvPae.  pWhgVLdATg  lsatCvQery  eYFPGFSGeE
Dros. EST 6  nGsyysYesl  PYAEPPtGDL  RFeaPEPyKq  kWSdlFdATK  tPvaClQ.wD  qFtPG.....
Consensus    GG-V-A-LGI  PYAEPPVGDL  RFRKPEP-K-  -WS--W-ATK  YPN-C-Q--D  --FPGFSGSE

             121                                                         180
Torpedo AChE MWNPNrEMSE  DCLYlNlWVP  sPrPKstT..  ..........  ..........  ..........
Human BuChE  MWNPNTDLSE  DCLYlNVWlP  APkPKNaT..  ..........  ..........  ..........
Dros. AChE   lWNPNTnvSE  DCLYlNVWaP  Akarlrhgrg  anggehpngk  qadtdhllhn  gnpqnttngl
Dros. EST 6  ..anklvgeE  DCLtvsVYkP  knskrNsfp.  ..........  ..........  ..........
Consensus    MWNPNT--SE  DCLY-NVW-P  AP-PKN-T--  ----------  ----------  ----------

             181                                                         240
Torpedo AChE .VMVWIYGGG  FysGSsTLDV  YnGKYLAyte  eVVlVSLSYR  VGAFGFLALh  Gs......qE
Human BuChE  .VLIWIYGGG  FqTGtssLhV  YdGKFLArVe  rVlvVSMnYR  VGALGFLALp  Gn......pE
Dros. AChE   plLIWIYGGG  FMTGSaTLDl  YnadlMAaVg  nVlvaSFqYR  VGAFGFLhLa  pempsefaeE
Dros. EST 6  .VvahlhGGa  FMfGaawqng  henvmre..g  kfllVklSYR  lGpLGFvstg  dr.......D
Consensus    -VLIWIYGGG  FMTGS-TLDV  Y-GK-LA-V-  -VI-VS-SYR  VGA-GFLAL-  G--------E

             241                                                         300
Torpedo AChE APGNVGLLDQ  RMALQWVhDN  lqfFGGdPKt  VTIFGESAGG  ASVgMhILSP  GSRdLFRRAI
Human BuChE  APGNmGLFDQ  qLALQWVqkN  lAAFGGnPKs  VTLFGESAGa  ASVsLhLLSP  GShsLFtRAI
Dros. AChE   APGNVGLWDQ  aLAlrWlKDN  ahAFGGnPew  mTLFGESAGs  sSVnaqLMSP  vtRgLvkRgm
Dros. EST 6  lPGNyGLkDQ  RLALkWlKqN  lAsFGGePqn  VlLvGhSAGG  ASVhLqMLre  dfgqLaRaAf
Consensus    APGNVGL-DQ  RLALQWVKDN  IAAFGG-PK-  VTLFGESAGG  ASV-L-LLSP  GSR-LFRRAI

             301                                                         360
Torpedo AChE LQSGSpNcPW  AsVSvaEgRr  RAvELGRNln  CNl.nsDeEL  lhCLReKKPQ  EIldVEwnVL
Human BuChE  LQSGSfNAPW  AvtSlyEARn  RtLnLakltg  Csr.EnETEl  lkCLRnKdPQ  ElLlnEafVv
Dros. AChE   MQSGtmNAPW  shmtsekAve  lgkaLlndcn  CNasmlkTnp  ahvMscmrsv  DaktlsVqqW
Dros. EST 6  sfSGnaldPW  ..VlqkgARg  RAFELGRNvg  CesaEdsTsL  kkCL.ksKPa  seLvtaVrkF
Consensus    LQSGS-NAPW  A-VS--EAR-  RA-ELGRN--  CN--E--TEL  I-CLR-KKPQ  E-L--EV-V-

             361                                                         420
Torpedo AChE PFdSlfrfsF  ..vPvIDGEF  FPtslEsMLn  sGnFKKTQIL  LGVNKDEGsF  FLLYGAPG.F
Human BuChE  PYgtplsvnF  ..gPtVDGDF  LtDmPDlLLe  lGqFKKTQIL  vGVNKDEGTa  FLvYGAPG.F
Dros. AChE   nsYSglls.F  PsaPtlDGaF  LPadPmTLMk  tadLKdydlL  MGnvrDEGTY  FLLYdfldyF
Dros. EST 6  llFSyvp.fa  PfsPvlEpsd  aPDallTqdp  rdvlKsgkfg  qvpwavsyvt  edgg.....Y
Consensus    P--S------F  P--P-IDG-F  LPD-P-TLL-  -G-FKKTQIL  -GVNKDEGT-  FLLYGAPG-F

             421                                                         480
Torpedo AChE SKDseSKIsR  EDFMsGvKls  vPhAnDLGLD  AVtLQYTDWm  DDNnGIKNRd  gLdDIVGDHN
Human BuChE  SKDnnSlltR  kEFqEGLKlf  FPgvsEFGkE  sllFhYTDWv  DDqrpenyRe  aLGDVVGDyN
Dros. AChE   dKDdatalpR  DkYLElMnNl  FgkAtqaerE  AllFQYTsWE  g.NpGyqNqq  qlGraVGDHf
Dros. EST 6  naallIKerk  sglvlddlNe  rwlelapyLl  fyrdtkTkkD  mDdysrKlkq  eylgnqrfdl
Consensus    SKD--SKI-R  --F-EG-KN-  FP-A---GLE  AI-FQYTDW-  DDN-G-KNR-  -LGD-VGDHN

             481                                                         540
Torpedo AChE vICPlMhFvn  KYtkFGNgTY  LYFFnHRaSn  LvWPEWMGVl  HGYEIEFVFG  LPLvkELNYT
Human BuChE  FICPaLEFtk  KFsEWGNnaF  FYYFeHRsSk  LpWPEWMGVM  HGYEIEFVFG  LPLerrdNYT
Dros. AChE   FtCPtnEYaq  aLaERGaSvh  YYYFtHRtSt  slWgEWMGVL  HGdEIEYfFG  qPLnnsLqYr
Dros. EST 6  esYseLqrlf  tdllFkNSTq  esLdlHRkyg  kspayayvyd  npaEkglaqv  LanrtDydFg
Consensus    FICP-LEF--  K--EFGNST-  -YYF-HR-S-  L-WPEWMGV-  HGYEIEFVFG  LPL---LNYT

             541                                                         600
Torpedo AChE aeEEavSRRl  mhyWAtFAKT  GNPNEphsqe  skWPLFttkE  QKFldLNtEp  mkVhqrLRvQ
Human BuChE  kaEEILSRsl  vkrWAnFAKy  GNPNEtqnns  tsWPvFkstE  QKYlTLNtEs  trlmtkLRAQ
Dros. AChE   pVErELgkRm  lsavleFAKT  GNPaqdgeeW  pnFskedpvy  ylFsTddkle  klargpLaAr
Dros. EST 6  tVhgDdy.fl  lfenfvrdve  mrPdEqllsR  nflnMLadfa  ssdngslkyg  ecdfkdsvgs
Consensus    -VEE-LSRR-  ---WA-FAKT  GNPNE-----  --WP-F---E  QKF-TLN-E-  ------LRAQ

             601                                                         657
Torpedo AChE mCvFWNqFLP  KlLnaTeTID  EAERqWKteF  HRWssYMMhW  KNQFdhY.Sr  hESCaeL
Human BuChE  qCrFWtsFFP  KVLemTGnlD  EAEWeWKagF  HRWnnYMMdW  KNQFndYtSk  KESCvgL
Dros. AChE   .CsFWNdYLP  KVrswaGTcD  gdsgsaslsp  rlqllglaal  lylcaal.rt  Krvf
Dros. EST 6  ekfqllalYl  daarlgsmwn  frklhe
Consensus    -C-FWN-FLP  KVL--TGTID  EAE--WK--F  HRW--YMM-W  KNQF--Y-S-  KESC--L
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FIG. 1.—An alignment of esterases. The GAP program of the University of Wisconsin Genetics Computer Group (Devereux et al. 1984) was used to align *Torpedo californica* acetylcholinesterase (Torpedo AChE), human butyryl cholinesterase (Human BuChE), *Drosophila melanogaster* acetylcholinesterase (Dros. AChE), and *D. melanogaster* esterase 6 (Dros. EST 6). Each sequence includes a signal peptide of varying length. References to the primary structures of these proteins are provided in the text. A consensus residue is indicated at each position if two or more of the residues are identical. Uppercase letters indicate residues in accord with the consensus; lowercase letters show amino acids that differ from the consensus. The absence of a consensus residue is indicated by a dash.

On the other hand, while His and Arg are both basic amino acids, their pK_a values differ greatly (6.5 for His and 12 for Arg).

Other possibilities for the basic residue in the charge relay are two His residues

that occur on the carboxy-terminal side of the conserved Ser at positions 506 and 521 in the consensus sequence, as suggested by Sikorav et al. (1987). Although there is no precedent for the catalytic His to be on the carboxy side of the Ser in the primary sequence of a serine hydrolase, the position of the catalytic Asp and His residues relative to the Ser in the primary structure is reversed in subtilisin, a serine protease unrelated to trypsin-like proteases (Carter and Wells 1987). In addition, Carter and Wells (1987) have shown that the function of the His in subtilisin can be partially replaced by a His residue in an appropriate position in a substrate. While the His at position 506 is conserved in all four esterases, the His residue at position 521 of the alignment is absent in EST 6. However, another His is now found nearby at position 543 in EST 6 which may have the same role. Some involvement of the imidazole group of a His in the catalytic mechanism of esterases is also suggested both by the pH dependence of the catalytic parameters of AChE (Rosenberry 1975) and by the observation that agents that modify imidazole groups inhibit AChE activity (Roskoski 1974). However, these observations could be explained by other steric requirements for a His rather than a direct involvement in the charge relay.

Other regions of strong conservation in the alignment shown in figure 1 either do not contain the appropriate conserved residue (e.g., positions 70–82), are near Cys residues involved in forming disulfide loops (positions 105 and 132, 331 and 343, and 483 and 602; MacPhee-Quigley et al. 1986), or likely are involved in substrate binding or specificity (region around the reactive Ser and the proposed anionic site, position 408; Sikorav et al. 1987). Also consistent with the proposed location of critical catalytic residues are the full sequence data for acetylcholine transferase, an enzyme believed to derive from AChE but that has lost any esterase activity. Despite >30% global identity with the *Torpedo* AChE, the consensus regions including both the Arg at position 220 and the His residues at 506 and 521 have been lost in the transferase (Mori et al. 1987).

Conclusions

For all these reasons, we now propose that the four esterases use either an Arg-Asp-Ser or a His-Asp-Ser charge-relay system. These alternatives differ from the charge-relay system found in the serine proteases in that the first differs in the identity of the basic residue and the second differs in the order of the basic residue in the primary sequence.

EST D is a substantially smaller molecule (molecular mass 34 kD) than the other four esterases (molecular mass ~60kD) and contains no peptides that correspond unambiguously to those surrounding any of the proposed catalytic residues. The most similar sequence has a three-in-eight match with the reactive serine region. No other region of similarity exists between EST D and the four other esterase sequences. We therefore suggest that the catalytic mechanism of EST D is unlikely to be the same as that of the other four esterases, even though all five enzymes are classified, on functional criteria, as B-type carboxylesterases.

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