Structural and functional studies of the metalloendopeptidase (EC 3.4.24.15) involved in degrading gonadotropin releasing hormone

M. J. Glucksman*[‡], M. Orlowski[‡] and J. L. Roberts*

*Fishberg Research Center for Neurobiology and [‡]Department of Pharmacology, Mount Sinai School of Medicine, New York, New York 10029 USA

INTRODUCTION

Converging biophysical and molecular biological techniques including genetic and computer aided engineering are being utilized in the study of zinc-metalloendopeptidase E.C. 3.4.24.15 (EP 24.15), a peptidase involved in the degradation of Gonadotropin releasing hormone (GnRH), the master regulatory decapeptide involved in reproduction. This 71 kDa enzyme rapidly cleaves the Tyr^5-Gly^6 bond in GnRH, the rate limiting reaction in hormone inactivation. EP 24.15 has been identified and isolated (1, 2) and the full length rat cDNA has recently been sequenced, and used to direct the expression of the functional 645 amino acid protein (3). The sequence of EP 24.15 shows no sequence identity with any known metalloendopeptidases beyond the commonly shared active site motif, -H-E-x-x-H-, found in this family of enzymes. EP 24.15 is a predominantly cytosolic enzyme that is stable, not glycosylated, and can be modeled with other globular proteins. Hydrophobic Cluster Analysis, (HCA) (4), is a heuristic algorithm ascertaining structural domains by detecting patterns of secondary structure and sequence homology in a two-dimensional analysis. This method has been used to determine if thermolysin may be used in modeling EP 24.15, and extending this analysis to enkephalinase and angiotensin converting enzyme two other members of the zinc-metalloendopeptidase family. Information gained from this study would be a step towards modeling substrate and inhibitor interactions to elucidate the conformation of this enzyme, as a probe for the function of this enzyme in vivo, and as a target for pharmacological intervention.

RESULTS AND DISCUSSION

Information about biochemically well-characterized proteins can be used for modeling homologous proteins to yield a testable model as was shown in the study of serine proteases (5). A candidate homologous protein to EP 24.15 is thermolysin, a bacterial zinc metalloprotease for which high resolution x-ray diffaction data are available (6). One dimensional alignments based solely on homology are not sufficient to establish relatedness, however, there is still a feasible route to modeling a protein from its sequence because new structures comprise motifs that have been identified and characterized in other related proteins. This approach would be Hydrophobic Cluster Analysis (HCA), which examines motifs in related proteins.

The difficulties with certain paradigms concerning the prediction of protein structure lies in the inability to ascertain pattern recognition and prognostication. Recently, novel approaches to secondary structure analysis designated as knowledge based predictions have been proposed (7), utilizing structural information from related proteins, and establishes rules to define a template for a structural element in an analogous protein. Whereas there are only a few arrangements of secondary structure motifs that yield stable globular domains in proteins, the sequence requirements from the primary structure that predetermine folding is unknown.

The sequences of EP 24.15 (3), thermolysin (6), enkephalinase (8) and angiotensin converting enzyme (9), all zinc metalloenzymes were initially aligned using the computationally quick algorithm, FASTA to search the sequence of EP 24.15 against our metalloenzyme database. The algorithm BESTFIT was then employed using a fixed common motif at functional domains and the active site to search for the best homologies trying several gap penalties and checking for the consistency of alignments. Though the percent identities between EP 24.15, enkephalinase and thermolysin are all ~20%, their similarity, taking into account conserved amino acid substitutions, are 40–44%. The linear one-dimensional algorithm alone is less reliable than combining these results with a two-dimensional method (HCA) which examines structural motifs and domains.

After results from the one-dimensional search, HCA was implemented on the above set of proteins. Maximizing amino acid alignments as a first approximation, a comparison of structural segments is made of the hydrophobic core of domains within globular proteins. Do-

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Address correspondence to Dr. Glucksman.

main elements shared within the metalloproteases are identified and then compared. A modified beta version kindly provided by Dr. J. P. Mornon, University of Paris, France, with the ability to do pairwise alignments was adapted by us to interface with graphics programs. A hydrophobic cluster is defined as the spatial grouping of hydrophobic residues on a two-dimensional helical net, with horizontal clusters representing alpha helical segments. The alignment of putative secondary structure elements by HCA begins at a fixed place where there is a
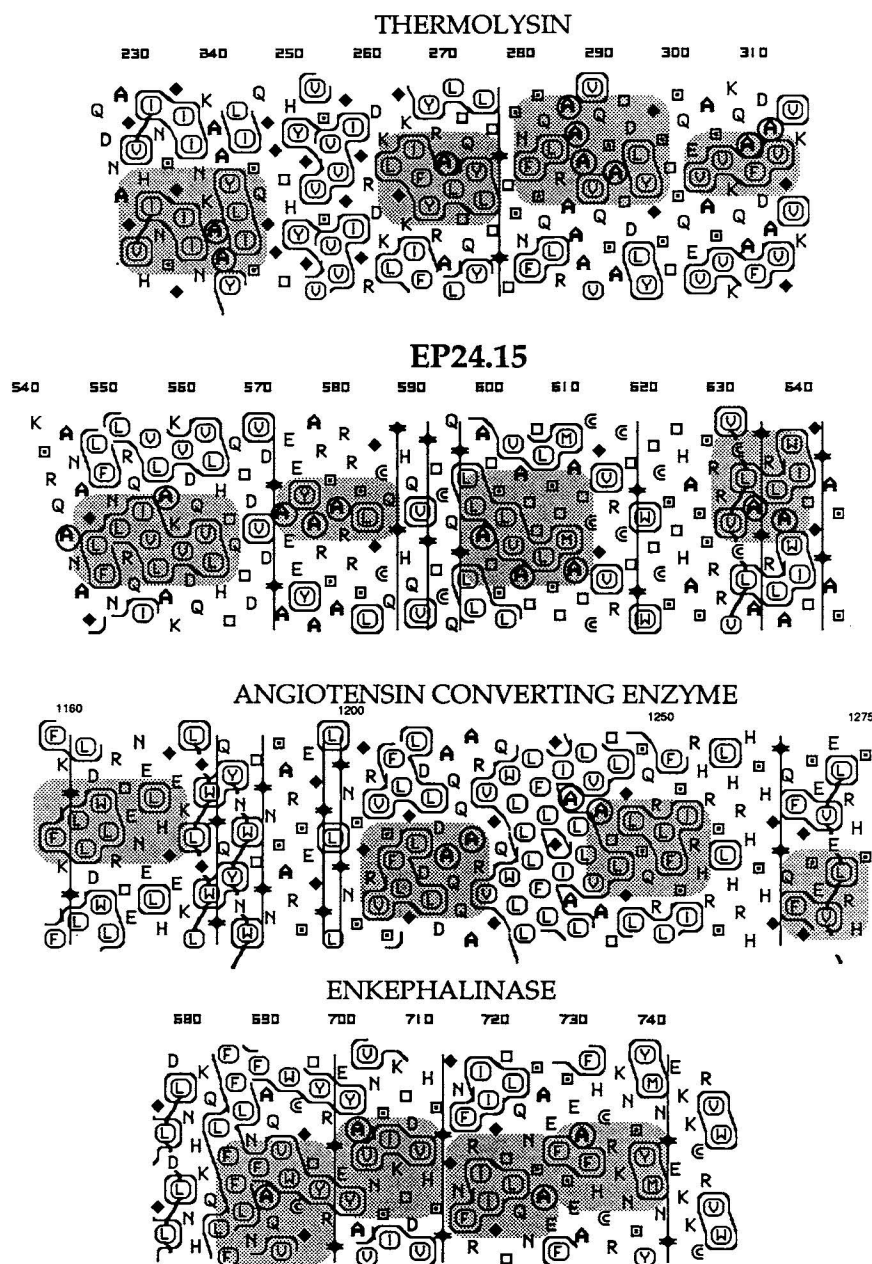

FIGURE 1 A comparison by Hydrophobic Cluster Analysis of the four helix bundle region at the carboxy terminus of thermolysin (*top*), whose structure has been determined by x-ray diffraction analysis and EP 24.15, enkephalinase and angiotensin converting enzyme. The regions of alpha helix are shaded. The helical regions assigned to thermolysin, are based on known secondary structure. The helical net is drawn in duplicate to assure continuity in the output. Symbols: star (*) with a vertical line, prolines; (◆), dark diamond, glycine; dotted and blank square, serine and threonine respectively. The one letter code for amino acids is used.

region of interest such as an active site or a hydrophobic cluster representing known secondary structure. Clusters are thus best conserved with respect to position and not sequence identity (4). The places that were chosen include the active site and an identified four-helix bundle at the carboxy terminus of thermolysin. This type of approach was used because it was shown to successfully compare thermolysin and enkephalinase, an EP 24.15 functionally related metalloendopeptidase (10).

As a demonstration of the method of Hydrophobic Cluster Analysis, two well-characterized domains were chosen in thermolysin, a structure solved by x-ray diffraction, and a comparison was made to see if there were analogs structural domains in EP 24.15, enkephalinase,
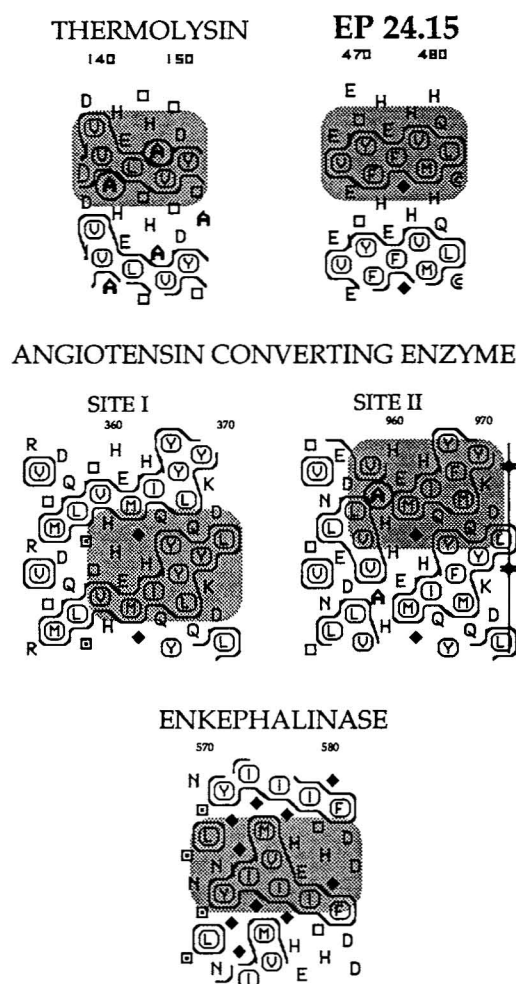

FIGURE 2 A comparison by Hydrophobic Cluster Analysis of the active catalytic site regions of thermolysin (*top*), whose structure has been determined by x-ray diffraction analysis and EP 24.15, enkephalinase and angiotensin converting enzyme. The regions of alpha helix are shaded. The key to residues is described in Fig. 1.

a mammalian metalloenzyme with substrate specificity similar to EP 24.15, and angiotensin converting enzyme, a mammalian enzyme with two putative zinc binding motifs. The carboxy terminal four alpha helix bundle domain identified by x-ray diffraction in thermolysin is compared to similar helix bundles in the related enzymes as shown in (Fig. 1). Similarly, the active site region was also examined to compare the putative alpha-helical conformation within these metalloproteases (Fig. 2).

A putative functional correlate to this study lies in the potential function of EP 24.15 in the regulation of blood pressure and for potential drug design by intervention in the renin-angiotensin system. Recently, in vivo, an administered EP 24.15 inhibitor was shown to lower blood pressure (11), presumably by inhibiting the degradation of bradykinin, a peptide substrate for EP 24.15. Inhibition of the two other metallopeptidases, included in this work, angiotensin converting enzyme and neutral endopeptidase 24.11, have been demonstrated to be antihypertensive agents.

There is excellent agreement with the helical regions predicted by HCA for thermolysin, compared with the actual determined secondary structure by x-ray diffraction (5). The four helix bundle (Fig. 1) and active site alpha helices (Fig. 2) are present in EP 24.15, enkephalinase and angiotensin converting enzyme and are homologous with thermolysin. There is some ambiguity in the placement of a fourth helix in the bundle of angiotensin converting enzyme, but the amino-most helix was placed in the most likely region based on the horizontal cluster.

To experimentally confirm the validity of the assumptions used in predictive schema, CD spectra of the native (wild type) EP 24.15 were collected (Glucksman et al., manuscript in preparation). These studies indicated that EP 24.15 is 36% alpha helical. The Chou and Fasman analysis (12) is an empirical prediction scheme which calculates the probability that certain amino acids will form secondary elements based on analysis of known globular proteins whose x-ray structures have been solved. This scheme indicated that EP 24.15 is 38%

alpha helical, a close agreement with the experimental results by the method of circular dichroism (see Table 1). Both analyses support that there is sufficient helicity to accommodate our HCA derived model.

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