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Commentary

Is "Somatic" Angiotensin I-Converting Enzyme a Mechanosensor?

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ABSTRACT

"Somatic" angiotensin I-converting enzyme (ACE) appears to be one of the evolutionary advances that made a closed circulation possible, and may have contributed to the Cambrian "explosion" of species approximately 540 million years ago. It also appears to be at the origin of a large number of common human diseases. A model is proposed in which the duplicated form of ACE ("somatic" ACE) functions as a mechanotransducer, defending downstream vessels and tissues from an increase in pressure. In the model, ACE senses shear stress (blood velocity) in regions of turbulent blood flow. An increase in shear stress strips an autoinhibitor tripeptide, FQP, from the N-terminal active site, thereby activating it. The C-terminal domain is constitutively activated by chloride. This model explains the clinical superiority of hydrophobic ACE inhibitors relative to hydrophilic ones.

INTRODUCTION

ANGIOTENSIN I-CONVERTING ENZYME (ACE; kininase II, dipeptidyl carboxypeptidase I, EC 3.4.15.1; SwissProt accession number P12821) is a metallopeptidase whose zinc-binding active site, HExxH, defines a superfamily of proteins (the "metzincins"), which contains 36 families of enzymes.[1] ACE occupies Family M2. [The single-letter amino acid code is used throughout this paper. A = alanine, C = cysteine, D = aspartic acid, E = glutamic acid, F = phenylalanine, G = glycine, H = histidine, I = isoleucine, K = lysine, L = leucine, M = methionine, N = asparagine, P = proline, Q = glutamine, R = arginine, S = serine, T = threonine, V = valine, W = tryptophan, and Y = tyrosine.] The two histidines (H) bind to Zn^{2+}, while the carboxylate group of aspartate (E) engages in a nucleophilic attack on the carbonyl carbon of the peptide bond to be hydrolyzed. Subsequent addition of a water molecule completes scission of the peptide bond.

Orthologs of ACE are expressed in every kingdom, including Archaea and Eubacteria (Table 1). Thus, the active site of ACE is as old as life itself (i.e., approximately 3.5 billion years old).

The ancestral ACE gene underwent a duplication at the origin of Chordata approximately 540 million years ago that was retained in all subsequent species, including humans. The ancestral form of the gene is still expressed in mammals during terminal differentiation of the

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Table 1. Comparative Molecular Evolution of ACE: Ancestral Gene Product and C-Terminal Domains ("C") in Cases Where the Gene Has Undergone Duplication

Species, accession number	*Active site*	*Approximate position*		
		74	*123*	*213*
Halobacteria (Archaea), P29143	H^{198} GT H	Y (99) P	(121)FSNLGP	T (209)***TD***P G
E. coli aminopeptidase N, P04825	H^{296} EYF H	N (78)FYT L	D (124)FVQ A	Y (229)F***Q***P V
Xanthomonas axonopodis, Q8PN56	H^{424} ELG H	A (73)FLP F	F (122)FDP G	Y (212)F***A***P M
Xanthomonas campestris, Q8PBK3	H^{424} ELG H	A (73)FLP F	F (122)FDA G	Y (212)F***A***P M
Lactobacillus lactis aminopeptidase N, P37897	H^{287} ELA H	V (72)THP D	L (81) FDP A	Y (214)F***K***R H
Saccharomyces cerevisiae Ala/Arg aminopeptidase, P37898	H^{300} ELA H	K (37)FQP	I (81) FDA I	V (305)F***E***P
Caenorhabditis elegans, Q18581	H^{531} SLLVQ	S (74)FTKLP	L (124)FDG T	Y (214)Y***E***P L
Buffalo fly, Q10715	H^{367} EMGH	V (74)FLP F	D (123)FDAPA	Y (219)F***E***P L
Cattle tick, Q17248	H^{387} EMGH	A (72)FLP F	F (121)FDG G	Y (214)Y***E***P L
ACE				
Chicken, Q10751, C domain	H^{886} EMGH	A (74)FLP F	D (123)FDP G	Y (213)F***E***P L
Mouse, P09470, C domain	H^{993} EMGH	A (74)FIP F	D (123)FDP G	Y (214)F***K***P L
Rat, P47820, C domain	H^{994} EMGH	A (74)FIP F	D (123)FDP G	Y (213)F***K***P L
Rabbit, P12822, C domain	H^{992} EMGH	A (74)FIP F	D (123)FDP G	Y (213)F***K***P L
Chimpanzee, Q9GLN7, C domain	H^{985} EMGH	A (74)FIP F	D (123)FDP G	Y (213)F***K***P L
Human, P12821, C domain	H^{988} EMGH	A (74)FIP F	D (123)FDP G	Y (214)F***K***P L

SwissProt accession numbers are provided. For ease of comparison of downstream tripeptides, a second numbering system is introduced, starting with the N-terminal-most H of the zinc-binding motif, HExxH, as number 1. For example, the numbering of human ACE according to SwissProt accession number P12821 is: HH^{390}EMGH...YF^{603}QPV...HH^{988}EMGH...YF^{1201}KPL. Renumbering the FxP tripeptides C-terminal to the two active sites yields the following: ...H^{390}EMGH...Y (603 − 390 = 213)FQP V... for the N-domain; and H^{988}EMGH...Y (1,201 − 988 = 214)FKP L for the C-domain. The middle amino acid in the FxP tripeptide at position approximately 213 has remained small (A) or changed (D, E, K) throughout most of evolution (see text).

spermatocyte into the spermatid, and so is called "testicular" or "germinal" ACE. The duplicated form of the gene is widely expressed elsewhere in the body, and so is called "somatic" ACE (Table 2).

Relative overactivity of ACE appears to be associated with many age-dependent common diseases in humans, including cardiovascular disease, cancer, and psychiatric disease.[2] The present paper attempts to explain why the duplication of the ACE gene was such a successful event in evolution, and how the duplicated gene product might contribute to disease.

WHEN DID THE ACE GENE UNDERGO DUPLICATION?

Gene duplication is common in evolution.[3] A duplicated gene product may afford better regulatory control than the ancestral protein, as in mammalian hexokinase. One-half of mammalian hexokinase retains a functional active site, while the other half acquired a regulatory function.[4] However, both domains of ACE have retained enzymatic activity,[5] similar to endothelin-converting enzyme, which, like ACE, functions as a homodimer.[6] If dimerization enables better regulatory control of ACE, then the mechanism is distinct from that of hexokinase.

The ancestral HExxH-containing gene, present in Halobacteria (members of Archaea, the Kingdom that predated Eubacteria; Table 1), underwent duplications several times before its stunningly successful duplication at the origin of Chordata (Table 3). One of the earliest duplications appears to have occurred in yeast (*Saccharomyces pombe*), a unicellular eukaryote whose last common ancestor with humans was about 1 billion years ago. Yeast DNA helicase contains two zinc-binding motifs that are duplicated in mirror-fashion: HEgnH . . . HaaEH (Table 2). Perhaps the unusual mirror duplication was important in changing the enzyme's function from proteolysis to unwinding DNA.

In Insecta, by contrast, the gene was duplicated in tandem (HExxH...HExxH), and retained proteolytic activity (Table 3). The mosquito (*Anopheles gambiae*), which last shared a common ancestor with humans approximately 430 million years ago, contains the ancestral gene as well as two tandemly duplicated versions of ACE (Table 3).

In the fruit fly (*Drosophila melanogaster*), which arose approximately 55 million years ago, some 260 million years after *Anopheles*, the ancestral ACE gene was copied at least six times.[7] After one of these duplications, a genomic sequence of 36.5 kb, which is not too large to behave as an intron, separated *Ance-2*, which is similar to the N-terminal domain of somatic ACE,[7] from *Ance-3*, which is similar to the C-terminal domain.[7] A tandemly duplicated protein would result if RNA polymerase II reads both transcripts in series. The *Drosophila* EST database does not contain such a transcript, but the EST database may lack approximately 50% of transcripts.[7]

None of the duplications in yeast or Insecta (Table 3) yielded the same tripeptides located

Table 2. Comparative Molecular Evolution of ACE-Like Proteins: N-Terminal Domains ("N") of Duplicated Gene Products

		Approximate position		
Species, accession number	*Active site*	*74*	*123*	*213*
Chicken, Q10751, N	H^{288} EMGH	A (74)FLP F	N (123)FDP G	Y (213)F**Q**P V
Mouse, P09470, N	H^{395} EMGH	A (73)FLP F	H (123)FDA G	Y (213)F**Q**P V
Rat, P47820, N	H^{396} EMGH	A (73)FLP F	H (123)FDA G	Y (213)F**Q**P V
Rabbit, P12822, N	H^{395} EMGH	A (73)FLP F	H (123)FDA G	Y (212)F**Q**P V
Chimpanzee, Q9GLN7, N	H^{388} EMGH	A (73)FLP F	H (123)FDA G	Y (214)F**Q**P V
Human, P12821, N	H^{390} EMGH	A (74)FLP F	H (123)FDA G	Y (213)F**Q**P V

Amino acid residues are renumbered as described in Table 1. SwissProt database accession numbers are provided. The ACE N-terminal domain in Chordata contains exclusively the tripeptide F**Q**P at the position approximately 213 amino acids C-terminal to the active site.

Table 3. ACE Gene Duplications Before Chordata

Species, accession number	Active site	Approximate position 74	123	213
S. pombe DNA helicase, Q9URU2				
N	H^{373} EGNH	S (76) FP C	F (108) FQH A	Y (139) F***A***D L
C	H^{945} AAEH	H(91) FYEDPQ	K (117) FDY C	Y (203) G***G***NL
Fruit fly				
Acer, Q24222	H^{375} ELGH	V (74) FLP F	D (123) FDP P	Y (219) F***E***P L
Ance-1, Q10714	H^{367} ELGH	V (74) FLP F	D (123) FDA P	Y (219) F***E***P L
Ance-2, Q9VJV2	H^{245} GEH	L (72) GFP Q	R (121) FFN M	Y (345) F***Q***P L
Ance-3, Q9VJV1	H^{535} EMAH	A (74) FLP F	P (131) GDP R	Y (186) F***A***P L
Mosquito				
Ancestral, GenBank EAA14500	H^{352} ELGH	V (74) FLP F	D (123) FDA P	Y (219) F***Q***P L
GenBank EAA09164				
N	H^{392} EMGH	A (74) FLP F	D (123) FDP G	Y (220) F***E***P L
C	H^{954} EMGH	A (74) FLP F	D (123) FDP G	Y (219) Y***K***P L
GenBank EAA14498				
N	H^{318} ELGH	V (74) FLP F	D (123) FDP P	N (210) F***LN*** E
C	H^{924} ELGH	V (74) FLP F	D (123) FDA A	Y (219) F***Q***P L

SwissProt accession numbers are provided except where the GenBank accession number is indicated. In yeast (*S. pombe*), the duplicated gene product functions as a DNA helicase rather than a protease. In the fruit fly (*D. melanogaster*), an *Ance-2*::*Ance-3* transcript[7] would result in *Ance-2* as the N-domain, containing the tripeptide F***Q***P 345 amino acids C-terminal to the active site, and *Ance-3* as the C-domain with F***A***P 186 amino acids C-terminal to the C-domain active site. None of the duplications above results in both the F***Q***P tripeptide at position approximately 213 in the N-terminal domain and a charged F***(E/K)***P tripeptide at position approximately 213 in the C-terminal domain, as in vertebrates (Table 2).

approximately 213 amino acids C-terminal to the two active sites as did the gene duplication at the origin of Chordata (Tables 1 and 2).

Apart from the duplications noted above, species before Chordata, such as invertebrates like the mollusk,[8] possess only the unduplicated, ancestral form of the ACE gene. Once the duplicated form of ACE appeared in the marbled electric ray (*Torpedo marmorata*),[9] it was retained in all subsequent vertebrate species. Interestingly, birds express only the duplicated form of ACE (Tables 1 and 2), suggesting that the duplicated form of the gene is an improvement on the ancestral form, making the latter dispensable.

"SOMATIC" ACE APPEARED AT THE SAME TIME THAT THE CIRCULATION BECAME CLOSED

Instead of the open circulation of insects and mollusks, which express the ancestral unduplicated form of ACE, all Chordata have a closed circulation completely lined by endothelial cells.[10] Before Chordata, the arterial circulation ended in tissue sinuses 10–20 μm in diameter.[11] Fluid is then collected in veins and returned to the heart.[12] In invertebrates, the circulating fluid is called hemolymph, since it shares characteristics of both blood (it contains formed elements such as red and white cells, as well as clotting factors and antibodies) and lymph (it percolates through tissue sinuses).

Regulation of open circulations can be quite complex, since flow is matched to the metabolic state of the perfused organs.[11] Systemic pressure, not regulatory complexity, seems to be the major difference between open and closed circulations. The pressure of an open circulation is limited to less than 10 mm Hg,[12] whereas in a closed circulation pressure can rise two orders of magnitude higher (e.g., 240 mm Hg systolic pressure in the giraffe).[13] In addition to opposing the force of gravity, making life on land possible for large animals, a significant advantage of higher blood pressure is higher blood velocity and shorter circulating time.

Evolution has been able to achieve homeostasis in ever harsher environments. Preservation of the *milieu intérieur* defies entropy, and requires sophisticated information manage-

ment within the organism. Homeostasis within the cell, as well as within multicellular organisms, requires efficient communication among different compartments. Homeostasis in more complex organisms can succeed only if the biochemical signals linking organs are transmitted quickly and at low cost. A high-pressure circulation with high blood velocity allows hormonal and metabolic signals to be transmitted quickly. As a mechanosensor, "somatic" ACE appears to have also kept the cost of the circulatory system to a minimum (see below).

Development of a closed circulation coincided with the Cambrian "explosion" in new species approximately 540 million years ago. In addition to the notochord, a closed circulation appears to be one of the key features that made the Cambrian "explosion" in new life forms possible. This paper's hypothesis is that duplication of the ACE gene in chordates resulted in a mechanosensor that made higher-pressure circulations possible.

SYSTEM REQUIREMENTS FOR A HIGH-PRESSURE CIRCULATION

To accommodate high pressure, the circulation must be closed. Otherwise an increase in arterial-sided pressure would force the circulating fluid out into tissue sinuses, with recapture by the venous limb being too inefficient to allow more than a few heartbeats. A hematoma is a good example of the limits of an open circulation. As every reader knows, resorption of blood extravasated by a high-pressure circulation takes days rather than seconds.

It is not sufficient merely to have a closed tubular system. The integrity of the vessel walls must be maintained so as to prevent rupture. In the face of chronically elevated pressures, vasoconstriction is not sufficient. The wall itself must be mechanically strengthened. Angiotensin II can do both: vasconstrict acutely, as well as stimulate smooth muscle cell hyperplasia and reduplication of the internal elastic lamina (arteriolar "onion-skinning"). Elastin, whose synthesis is stimulated by angiotensin II, also appeared when the circulation became closed, further enabling higher driving pressures.[13]

As in any system of pipes transporting a fluid, an increase in upstream pressure must be sensed and resisted so that it is not passed on downstream. Otherwise, the pipeline may break. In man-made systems, this is done with a combination of flow and pressure sensors and computer-controlled valves; when pressure increases somewhere in the pipeline, valves upstream are closed so as to lower the pressure in the system. This paper proposes that "somatic" ACE performs this crucial sensing and effector function in the closed circulation of vertebrates.

Such control is especially important in living organisms, in which the mechanical (e.g., heart) or chemical (e.g., kidney) workload of an organ is determined by the rate of blood flow into that organ. Similarly, an organ's metabolic activity cannot be allowed to exceed its fuel supply, or else ischemia and necrosis of the organ will result.

For example, if more blood is delivered to the heart than it can pump, mechanical heart failure results, with pulmonary edema, hypoxia, and death. If more blood is delivered to the kidney and filtered at the glomerulus than downstream elements of the nephron can reabsorb, then rapid loss of the body's water and salt would result. In the extreme, the body elects to shut down blood flow to failed kidneys and consign them to apoptosis rather than risk the loss of extracellular fluid (manuscript in preparation).

As a rule, organs exposed to the arterial circulation exhibit some degree of autoregulation: Blood flow is kept constant despite changes in systemic perfusion pressure. Some organs, such as the kidney and brain, exhibit extraordinary constancy of blood flow in the face of huge variations in perfusion pressure. Other organs can tolerate wider fluctuations in blood flow (e.g., the heart). This paper's hypothesis is that duplication of the ACE gene enabled better autoregulation than any previous mechanism.

WHAT IS THE MECHANICAL SIGNAL FOR AUTOREGULATION?

Blood flow is the product of blood volume times blood velocity. Despite the argument

above, surprisingly, blood flow is not kept constant, but rather one of its components, blood velocity.[14]

Blood moving in a tube creates shear stress at the vessel wall because of the "no-slip" condition. Under conditions of laminar flow, blood velocity is high in the midline of the vessel but must decrease to 0 at the vessel wall. The endothelial cell glycocalyx, made up of complex sugars of glycoproteins and glycolipids embedded in the endothelial cell plasma membrane, creates an unstirred layer of water molecules bound tightly by carbohydrate moieties. This unstirred layer pushes the point of 0 blood velocity away from the vessel wall towards the midline. In a 10-μm-diameter arteriole, the unstirred layer may occupy a 1-μm ring next to the vessel wall.[15]

As a result of the "no-slip" condition, there is a radial gradient of blood velocities within the vessel, with the highest velocity in the midline. Blood velocity decreases to 0 at the unstirred layer some distance r from the midline. Although the velocity gradient is continuous, it can be approximated by nested columns of blood of width dy moving at different speeds (Fig. 1).

A mechanical shearing force arises when columns of fluid move past one another at different speeds. Shear stress is given by the expression:

$$\tau = \mu\ (dV/dy) \tag{1}$$

where τ is shear stress, dV/dy is the change in velocity V with radial distance y (i.e., the velocity gradient), and μ, the proportionality constant, is blood viscosity.

Velocity increases from 0 in a parabolic fashion (Eq. 2). The shear stress (τ) is therefore maximal at the vessel wall where the velocity changes the most. Shear stress is least near the midline. The velocity (V) at any particular point y in the vessel cross-section is given by the following formula:[16]

$$V_y = \Delta P\ (r^2 - y^2)/4\ \mu l \tag{2}$$

where ΔP is the driving pressure or pressure drop along a blood vessel of length l and radius r, y is the radial distance from the midline of the vessel towards the vessel wall, and μ is again the blood viscosity. At the vessel wall, $y = r$, the $(r^2 - y^2)$ term becomes 0, so $V_y = 0$, satisfying the "no-slip" condition.

Equation 2 says that blood velocity anywhere within the vessel's lumen, V_y, is directly proportional to driving pressure. So the higher the systemic blood pressure, the faster that blood will circulate, and the faster that bloodborne messages will be delivered.

Experimentally, it is observed that blood velocity V is kept constant.[14] Assuming blood vis-

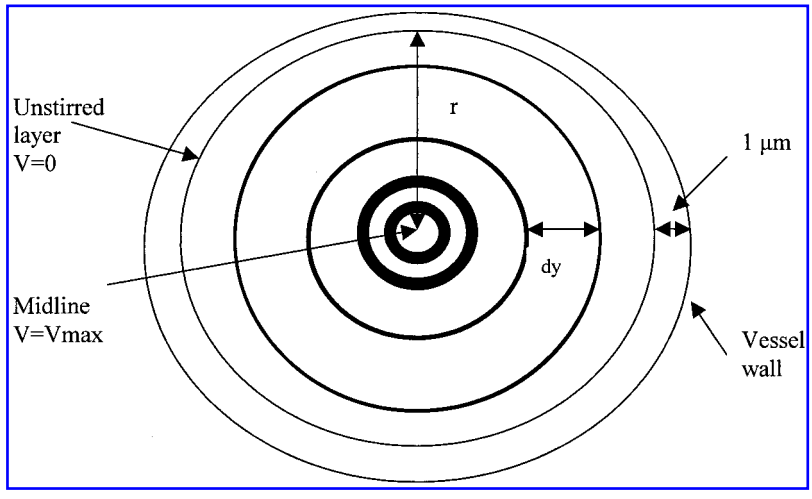

FIG. 1. Cross-section of an arteriole of diameter approximately 10 μm. The velocity of each column of blood is suggested by the thickness of the line surrounding the fluid column. In the midline, the velocity is highest; at the vessel wall, the velocity is required to be 0 by the "no-slip" rule. Velocity changes in a parabolic fashion with distance from the vessel wall, making the velocity gradient steepest near the vessel wall.

cosity μ is also kept constant (this is a bad assumption in diabetes, as we shall explore below), then shear stress τ will be kept constant also.

This finding was predicted theoretically in 1926, and is referred to as Murray's law. To minimize the energy cost of a closed circulatory system, Murray's law states that shear stress at the vessel wall should be equalized throughout the circulation.[16–18] Thus, shear stress is the mechanical signal upon which the closed circulation is built.[19–21]

HOW IS SHEAR STRESS KEPT CONSTANT?

The problem with a high-pressure circulation is that the pressure gradient (ΔP in Eq. 2 above) is not constant, but can easily vary by as much as 50%. Systemic blood pressure can vary on a beat-to-beat basis depending on heart rate, myocardial contractility, resistance of either upstream or downstream vessels, degree of physical exertion, extracellular fluid volume, etc. All of these variables are under complex neural and hormonal control.

For autoregulation to obtain, then blood velocity (V_y in Eq. 2) must be kept constant despite changes in driving pressure ΔP. In general, vasoconstrictors resist pressure increases, and vasodilators resist decreases in pressure. Since ACE and angiotensin II are concerned with combating pressure increases, we shall consider only this case.

If ΔP rises, then blood velocity V_y can be kept constant only by decreasing the term $(r^2 - y^2)$ (i.e., by decreasing r^2). If pressure (ΔP) doubles, then the radius r need be decreased by only $\sqrt{2}$, or about 1.4. This suggests that vasoconstriction will be able to keep up fairly easily with any acute rise in perfusion pressure.

Let us see what happens to blood flow as a result of the circulatory system's keeping blood velocity constant rather than flow. Blood flow (Q) is related to the fourth power of the radius according to Poiseuille's equation:[22]

$$Q = \Delta P \pi r^4 / 8\ \mu l \qquad (3)$$

where the symbols are defined as in Eq. 2.

If pressure (ΔP) increases twofold, blood velocity V at any point y within the vascular lumen (V_y) will immediately increase twofold according to Eq. 2. As a result, the velocity gradient within the vessel will increase, as will the shear stress (Eq. 1). The increase in mechanical shearing forces will be sensed (see below), resulting in a $\sqrt{2}$-fold reduction in vessel radius and restoration of blood velocity to its previous value despite the twofold higher driving pressure (ΔP).

Blood flow will increase by a factor of 2 when pressure ΔP is doubled (Eq. 3). But the response of vasoconstriction will decrease flow by a factor of $(\sqrt{2})^4 = 4$ according to Poiseuille's equation[22] (Eq. 3). The net effect on blood flow ($\times$ 2/4) will be a reduction in half. Blood flow is kept more constant than this because of considerable dampening in the system,[13] and the existence of a powerful vasodilatory arm.

THE "MYOGENIC RESPONSE"

Vasoconstriction in response to increased perfusion pressure (ΔP) occurs within approximately 5 s, and is referred to as the "myogenic response."[23] This response requires the presence of both endothelial and smooth muscle cells. About half of this response appears to be due to the opening of stretch-activated calcium channels.[24] The other half appears to be due to a G protein-coupled signal,[24,25] which could easily be mediated by the angiotensin II type 1 receptor.[26]

I propose that the origin of the G protein-coupled signal for the "myogenic response" is activation of endothelial ("somatic," duplicated) ACE, and that only the duplicated form of vertebrate ACE can function as a mechanotransducer. The appearance of "somatic" ACE coincided precisely with the appearance of the closed circulatory system. This unique function could explain the dramatic evolutionary success of the duplicated molecule.

"Somatic" ACE is expressed in areas of high fluid flow in mammals, including endothelial cells lining the vasculature, the brush border membrane of proximal tubular cells in the kidney, the choroid plexus where cerebrospinal fluid is synthesized, the ciliary epithelium of the eye, and the brush border of the jejunum.

ACE is located on the plasma membrane of endothelial cells, and so is in an ideal physical position to "sense" blood velocity (Figs. 2 and 3). ACE is also in an ideal biochemical position to serve as a mechanotransducer, translating changes in mechanical force into changes in concentration of important biochemical signaling molecules. The biochemical effect of ACE activation will be a multiplicative systems gain in favor of vasoconstriction,[30,31] since ACE not only activates a vasoconstrictor (angiotensin II), but also degrades a vasodilator (bradykinin). Both angiotensin II and bradykinin are located at the beginning of multiple signaling cascades,[26,32] further amplifying the effect of ACE activation.

The role of angiotensin II is to maintain the integrity of the circulatory system on both a short- and long-term basis. For example, angiotensin II causes vasoconstriction immediately by itself as well as by stimulating expression within 30–60 min of endothelin, which is an even more potent vasoconstrictor; angiotensin II activates thrombin to initiate clotting as well as stimulating expression of genes in the clotting cascade; and angiotensin II stimulates fibrosis through expression of the transforming growth factor β-1 gene.

In the intermediate term (hours to days), angiotensin II leads to smooth muscle cell hypertrophy and hyperplasia, and expression of elastin.[13]

The result of chronic signaling by angiotensin II action can be seen in the "onion-

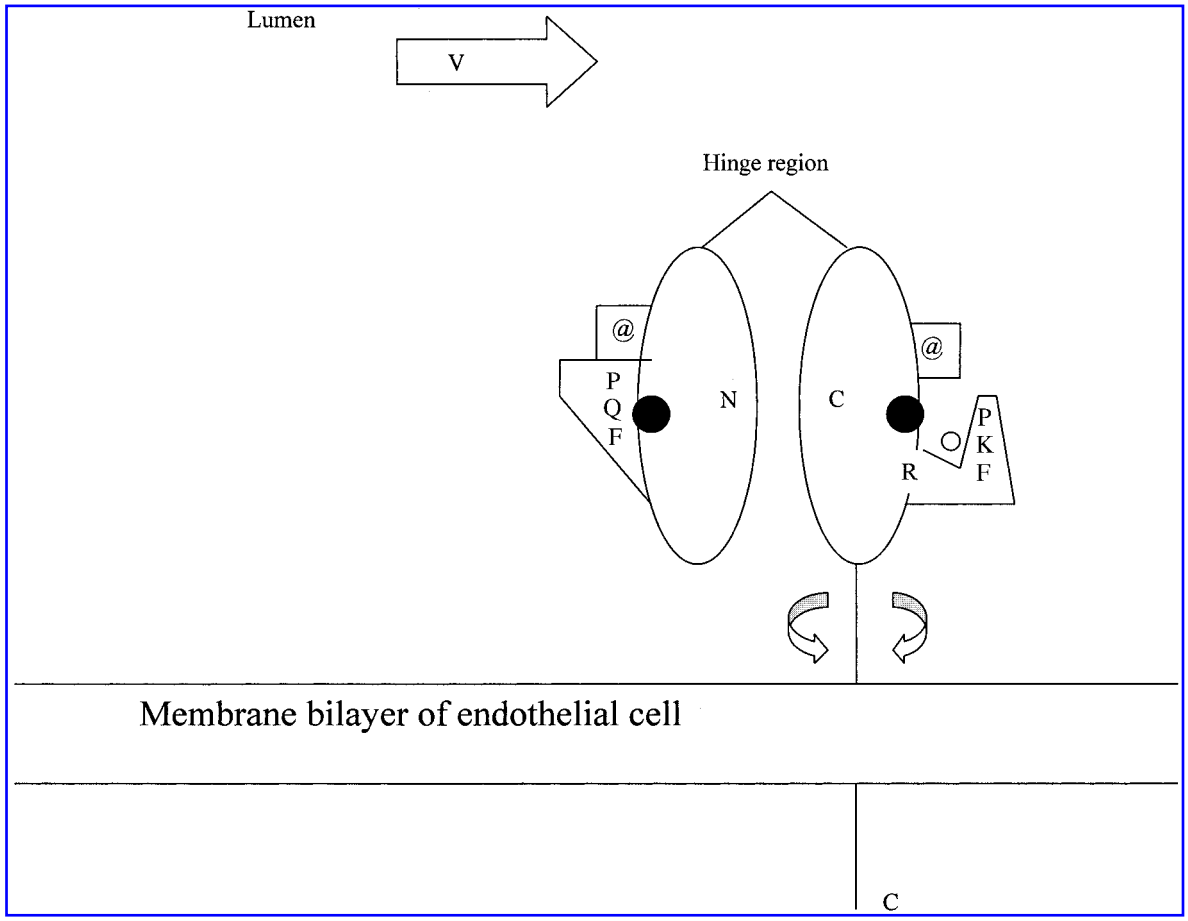

FIG. 2. Cartoon structure of "somatic" ACE (a virtual homodimer) under laminar flow conditions. Large arrow with *V*, blood velocity vector. The N-terminal active site is occluded; the C-terminal active site has been opened by chloride ion. Solid circle, zinc ion; open circle, chloride ion; @, "stop" feature at active site preventing protrusion of more than two or three amino acids past the active site. N, N-terminal domain; C, C-terminal domain; R, arginine 1,098.[27] The hinge region consists of amino acids 645–658 as numbered in SwissProt 12821.[28]

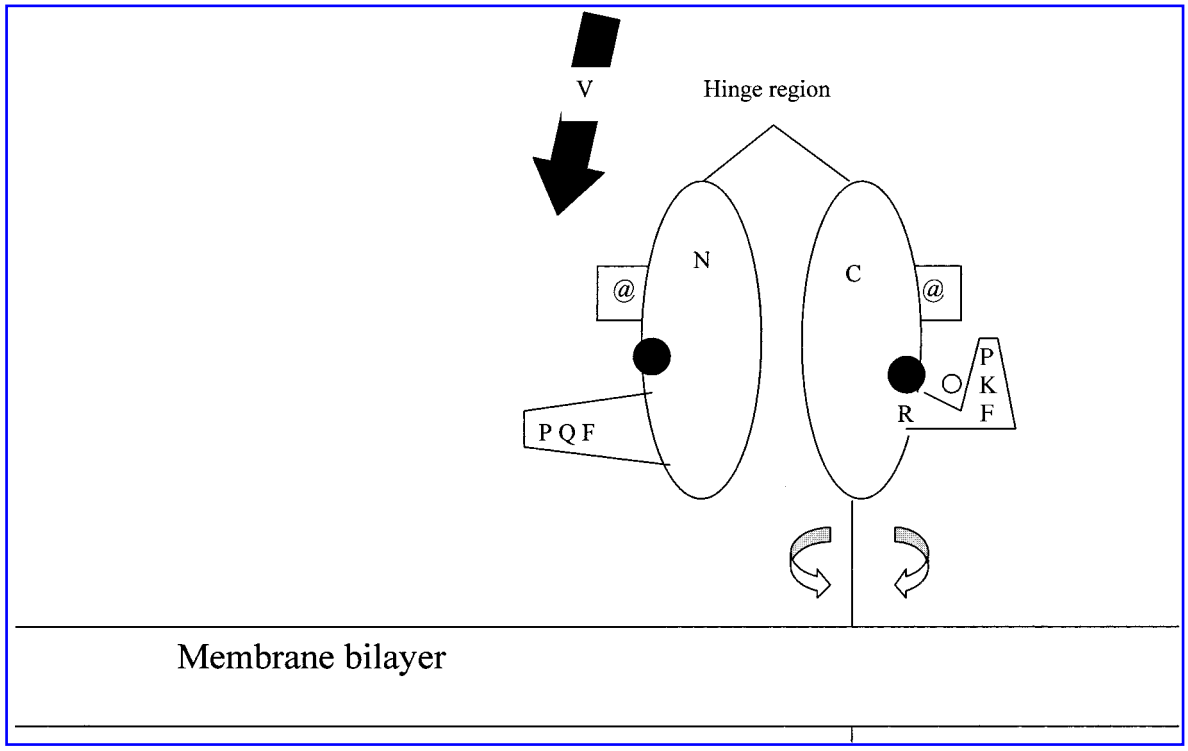

FIG. 3. Cartoon structure of "somatic" ACE in a region of turbulent flow. The N-terminal active site has been opened by a mechanical shearing force. The vector of blood velocity (large black arrow with *V*) is temporarily directed towards the vessel wall and at precisely the correct angle to force open the N-terminal active site. The FQP tripeptide is shown displaced from the N-terminal active site. The force required is expected to be less than 12 pN.[29] Black circle, zinc ion; open circle, chloride ion; @, "stop" feature at active site preventing extension of more than three C-terminal amino acids past the active site. N, N-terminal domain; C, C-terminal domain; R, arginine 1,098.[27] The hinge region consists of amino acids 645–658 as numbered in SwissProt 12821.[28]

skinning" of arterioles in patients with chronic hypertension. These arterioles display smooth muscle cell hyperplasia and a much thickened and reduplicated internal elastic lamina, with fibrosis. Angiotensin II can produce all of these changes.[26]

In the long term (decades), angiotensin II directs replacement of the endothelium and smooth muscle cells by the equivalent of inelastic cement, in the form of calcified collagen fibers. This involves apoptosis of endothelial cells in the intima and smooth muscle cells of the media,[33,34] with fibrosis mediated by transforming growth factor β-1,[20] and new bone formation[35] mediated by bone-specific proteins such as osteopontin.[36,37]

ACE is thus the guardian of vessel wall integrity. The clinical consequence for humans, especially as they age (manuscript in preparation), is that angiotensin II promotes sclerosis of large and small vessels, which is accelerated in diabetes (see below).

WHERE IN BLOOD VESSELS DOES ACE SENSE SHEAR STRESS?

ACE cannot sense changes in shear stress in regions of laminar flow. The unstirred layer next to the vessel wall, which may be about 1 μm thick,[15] is roughly 100 times larger than the height of an ACE molecule, since a globular type I integral membrane protein like ACE may protrude only approximately 10 nm from the plasma membrane of the endothelial cell. Its heavy glycosylation (40–50 kDa) means that ACE has a tree full of immobilized water molecules. Indeed, ACE is one of the glycoproteins that make up the endothelial cell's unstirred layer (the "glycocalyx").

Under laminar flow conditions, all transport occurs by diffusion rather than convection within the unstirred layer. Fluid velocity is always 0, and there are no shearing forces. Shearing forces begin approximately 1 μm away from the endothelial cell plasma membrane, a huge distance in molecular terms.

Apart from laminar flow, the only other flow regime encountered in the circulation is turbulent flow. (In this discussion, turbulent flow is used synonymously with "disturbed flow.")[38] In turbulence, the direction of the blood velocity vector changes rapidly with time, often chaotically.[39] The direction of blood velocity may change on the order of 0.1 s, and will often have a component perpendicular to the vessel wall. Such a velocity component would expose the ACE molecule to mechanical shear forces and convective flow.

WHAT IS THE EXPERIMENTAL EVIDENCE THAT ACE IS ACTIVATED BY TURBULENT FLOW?

Arterial endothelial ACE activity is normally low but increases in regions of atherosclerosis, suggesting a positive feedback loop.[40]

ACE is also present on the plasma membrane of T lymphocytes and macrophages.[40] Activated ACE on T lymphocytes can be found at the corner of coronary plaques.[41] Interestingly, the corners of the plaque are the only regions in the coronary profile where flow is turbulent; the rest of the profile enjoys laminar flow (Fig. 4). My hypothesis is that T-lymphocyte ACE represents an amplified view of what normally occurs with endothelial cell ACE.

In this scenario, turbulent flow activates ACE present on the endothelial cell membrane at the corners of the plaque. Angiotensin II is generated locally, and serves as a cytokine to attract circulating T lymphocytes and macrophages to the corners of the plaque.[42] In addition to attracting monocytes, locally generated ACE induces ACE expression by the monocytes themselves, generating an additional positive feedback loop.[43] Activation of monocyte ACE by turbulent flow then amplifies the amount of locally produced angiotensin II. Additional metalloproteases are induced (e.g., matrix metalloproteinase-1),[44] eventually leading to lysis of one corner of the fibrous cap and exposure of the underlying plaque. Acute thrombosis ensues, leading to coronary or cerebral infarction. This scenario causes death in two-thirds of Americans.

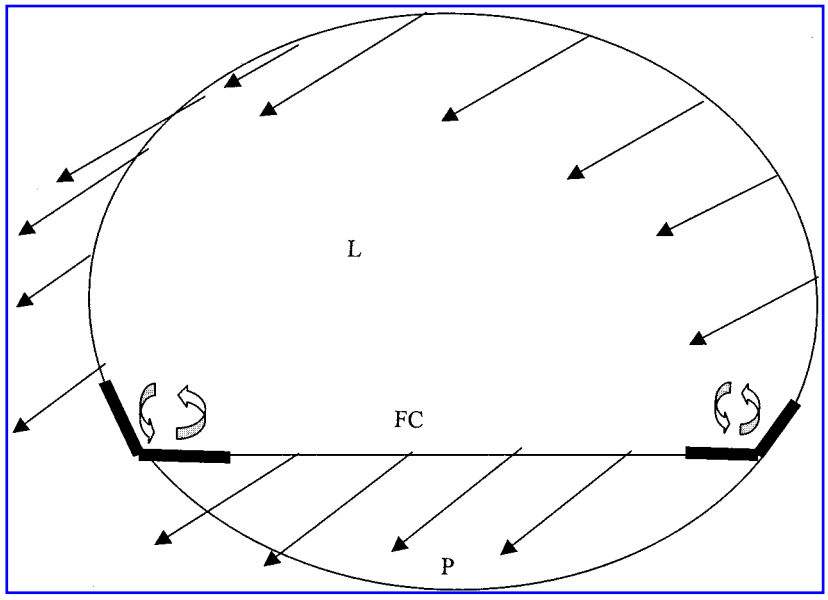

FIG. 4. Diagram showing where angiotensin II is deposited in the profile of a coronary artery containing a plaque (P).[41] Thick black lines, deposition of angiotensin II and location of activated ACE; FC, fibrous cap; L, lumen. Note laminar flow everywhere except the corners where the FC adheres to the vessel wall; in this region, the flow is turbulent (curved arrows).

Additional support for this hypothesis is the well-described formation of plaques downstream of vessel bifurcations, where flow is turbulent.[38]

HOW DOES ACE SENSE THE MECHANICAL SIGNAL?

If a shearing force in a regime of turbulent flow is the signal, how is it sensed by the ACE molecule? And why can only the duplicated form of ACE sense it, and not the ancestral, single domain still preserved on the human spermatid ("testicular" ACE)?

We have seen above how closed circulations must (and do) keep blood velocity constant. An increase in driving pressure increases blood velocity in areas of both laminar and turbulent flow, although the precise relationship between pressure gradient ΔP and velocity V_y is usually too complex in regions of turbulent flow to allow a precise mathematical solution. We have already seen how ACE in regions of laminar flow cannot sense a change in blood velocity happening hundreds of molecule-lengths above it. In regions of laminar flow, binding of substrate to ACE will continue to be diffusion-limited, and hence quite slow.

But in areas of turbulent flow, the blood velocity will at times be directed perpendicular to the vessel wall, resulting in convection replacing diffusion as the mode of delivery of substrate to ACE. Convective transport is many orders of magnitude faster than simple diffusion.[22]

Although increased blood velocity will increase convective transport to ACE molecules located in regions of turbulent flow, this cannot be the entire signal. Otherwise, there would have been no need for the gene to have undergone tandem duplication. Turbulence will bring just as much substrate to the ancestral, C-terminal domain, which is fully active above 50 m*M* chloride.

Hydrophilic ACE inhibitors such as enalapril,[45] which bind preferentially to the C-terminal active site,[46,47] have a pronounced clinical effect, suggesting that convective transport in areas of turbulence is important for increased local production of angiotensin II. But dramatically more clinical benefit is seen when hydrophobic ACE inhibitors are used.[48] Hydrophobic ACE inhibitors like ramipril and quinapril[45] can block both the N- and C-terminal domain active sites, suggesting that convective transport is not the whole story, and activity of the N-terminal domain is clinically significant.

Apparently, more amplification of angiotensin II was required than could be achieved by convective transport alone. Angiotensin II degradation is rapid, occurring within seconds, on a similar time scale as the rapid local activation and degradation of the proteins involved in the clotting and complement cascades. Increasing substrate delivery by convection evidently did not produce a sufficient increase in occupancy of angiotensin II receptors. In addition to increased local substrate concentration due to convection, a second active site had to be brought into play (see below).

This is admittedly a somewhat circular argument, since I am using the gene duplication in closed circulations as evidence that the C-terminal, ancestral form of ACE was inadequate to the task. However, every closed circulation expresses the duplicated form of ACE, and none expresses the unduplicated form of the gene. Indeed, the chick is an example of a species that has dropped the ancestral, single domain form of ACE altogether and expresses only the duplicated form (Tables 1 and 2). Whether a closed, high-pressure circulation could exist without the duplicated form of ACE remains to be shown.

WHAT IS THE MAGNITUDE OF THE FORCES INVOLVED?

In turbulent flow, the direction of the velocity vector changes on a beat-to-beat time scale (i.e., over less than 1 s). I hypothesize that a mechanical shearing force disrupts hydrophobic intramolecular bonds within the ACE molecule, exposing the N-terminal active site to solvent and hence substrate (angiotensin I and bradykinin).

For an idea of the magnitude of the force involved, approximately 12 pN of force is required to tear apart a "leucine zipper" coiled-

coil consisting of 31 amino acids in the yeast transcription factor GCN4 homodimer.[29] The hydrophobic forces involved in binding the FQP tripeptide to the N-terminal active site closed are likely to be smaller.

Shearing forces of 15–25 dynes/cm^2 are sufficient to produce biochemical changes in endothelial cells that reflect an increase in local angiotensin II levels.[19,20] Shearing forces of this magnitude are present under laminar flow conditions in arterioles and capillaries.[49] However, wall shear stress in large arteries, which is 10 dynes/cm^2 in laminar flow, can be further increased by turbulence.[49]

Direct rather than tangential exposure to blood pressure may be the more relevant pressure to consider. Mean systemic pressure of 100 mmHg exerts a force of approximately 1 pN on a 10 nm diameter-sphere. There may be a marked leverage effect by the glycocalyx tree, as well.

The magnitude of the shearing force depends on the degree of turbulence, which in turn depends on vessel configuration (nearness to a vessel bifurcation, degree of vessel curvature).[38] Higher blood pressure will result in higher blood velocity, a steeper velocity gradient, and increased shearing force, activating more ACE molecules throughout the vascular tree. Since shear stress also depends on viscosity (Eq. 1), a higher blood sugar concentration in diabetic patients will also result in activation of more ACE molecules throughout the vascular tree.

Let us examine the magnitude of the effect of diabetic hyperglycemia on shear stress. Ignoring the contribution of serum proteins, blood viscosity is proportional to blood osmolality. The latter can be approximated as

$$\text{Osm} = 2[\text{Na}^+] + [\text{glucose}]/18 + \text{BUN}/2.8 \quad (4)$$

where BUN represents blood urea nitrogen.

Doubling glucose from 100 mg/dL to 200 mg/dL will increase osmolality, and hence viscosity (μ) by only about 2%, since the serum sodium concentration is responsible for approximately 95% of blood osmolality. We have seen above how doubling blood pressure will double shear stress. Blood glucose concentration thus appears to have only approximately 1% (2%/200%) of the effect of blood pressure on increasing shear stress at the vessel wall. This may partially explain why it has taken decades to show any clinical benefit of strict glycemic control.

HOW DOES ACE ACT AS A MOLECULAR MECHANOSENSOR?

Detailed knowledge of the structural basis for ACE's behavior as a mechanosensor must await crystallization of the duplicated holoenzyme. However, insight into mechanism can come from comparison of the N- and C-terminal domains [i.e., comparative molecular evolution (Tables 1–3)].

In the design of ACE inhibitors, the active site of ACE has been assumed to be similar to that of thermolysin and other bacterial carboxypeptidases.[50,51] The active sites of thermolysin,[52] a carboxypeptidase from Archaea,[53] and a neutral protease from *Bacillus cereus*[54] all consist of a deep groove with the zinc-binding HExxH moiety at the bottom of the groove. In thermolysin, the walls of the groove are an α-helix on one side and a β-sheet on the other. The C-terminal tail of the substrate appears to feed into the groove until it encounters a "stop," akin to a fence on a table saw (Fig. 5). The aspartate (E) of the active site briefly binds to the carbonyl carbon, and the C-terminal dipeptide is released. A water molecule is added, releasing the shortened oligopeptide from the active site.

There is an ongoing search for a unique substrate that the N-terminal domain but not the C-terminal active site can hydrolyze, such as angiotensin (1–7).[55] Angiotensin (1–7) is cleaved by the N-terminal active site, but inhibits the C-terminal active site.[56] Angiotensin (1–7) is a pressor in volume-depleted but not euvolemic SHR rats,[57] and has a very weak pressor effect in humans.[58]

No physiologically compelling unique substrate for the N-terminal active site has yet been found. Given what is already known about angiotensin II and bradykinin, it appears that they are the physiologically most relevant substrates to consider.

ACE inhibitors in clinical use have the general structure of a tripeptide consisting of FxP,[50,51,59] where x = alanine (A) [i.e., FAP (as in enalapril)],[51] x = aspartate (E), or x = lysine

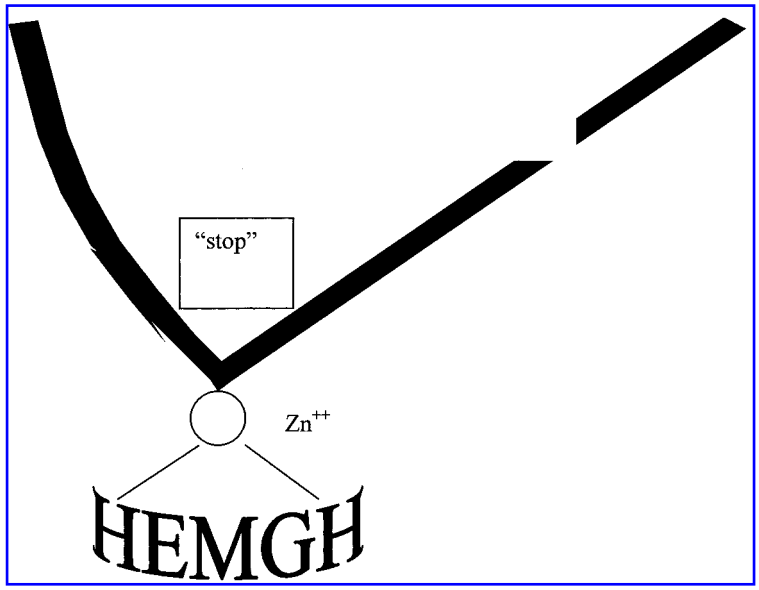

FIG. 5. Active site of ACE, based on its presumed similarity to thermolysin.[52] View from the substrate's C-terminal amino acid as the substrate docks with the active site.

[i.e., FKP (as in lisinopril)[51]].[59] Both enalapril and lisinopril bind preferentially to the C-terminal active site.[46,47] The N-terminal active site has a different affinity for some inhibitors, such as RXP 407,[60] and some substrates [e.g., angiotensin (1–7)].[56] However, as currently measured (i.e., in the absence of turbulent flow), catalytic efficiency of both active sites is similar towards angiotensin II and bradykinin.[61]

Inspection of the primary sequence of ACE shows that it contains three FxP tripeptides C-terminal to the HExxH active site, located approximately 74, approximately 123, and approximately 213 amino acids downstream (Tables 1 and 2). The tripeptides at positions approximately 74 [F(I/L)P] and approximately 123 [FD(G/P/A)] show no consistent pattern between C-terminal and N-terminal domains (Tables 1 and 2).

The tripeptide at position approximately 213, however, shows remarkable consistency. In the ancestral molecule, the tripeptide consists of FxP, where x is a charged (K,E) or a neutral (e.g., FQP in *Escherichia coli* aminopeptidase N and FAP in *Xanthomonas* species) amino acid (Table 1). A charged amino acid was retained in the C-terminal domain of all vertebrate species sequenced to date [Table 1; (~213) F(E/K)P].

In sharp contradistinction, the N-terminal tripeptide at position approximately 213 is FQP in all vertebrate species (Table 2). In other words, the middle amino acid changed from a charged residue (E/K) to a neutral one (Q).

Given their similarity to commercial ACE inhibitors, the FKP and FQP tripeptides may serve as autoinhibitors, similar to the calmodulin-like autoinhibitor in the N-terminal domain of Ca^{2+}-ATPase.[62] When the autoinhibitor is replaced by calmodulin, Ca^{2+}-ATPase becomes active. By analogy, each active site of ACE may bind an ACE inhibitor contained within the same domain. The auto-ACE-inhibitor must be released before substrate can gain access to the active site.

The C-terminal active site is exposed by chloride,[63,64] which can form a salt bridge between the positively charged lysine (K) of the FKP tripeptide[65] and arginine 1,098.[27] The size of the anion is important for this intramolecular rearrangement, since fluoride and bromide can substitute for chloride, but nothing larger,[63] suggesting that the anion fits into a pocket of defined dimensions (Fig. 3).

Since it is uncharged, the glutamine of the N-terminal FQP tripeptide cannot interact ionically with chloride. This may explain why the N-terminal active site does not undergo

marked chloride activation.[61] The N-terminal active site is fully activated in the presence of only 5 m*M* chloride. This may be due to charge neutralization of a positively charged histidine (H) in HFDAG approximately 122 amino acids C-terminal to the active site (Table 2).

The N-terminal active site preferentially binds hydrophobic ACE inhibitors, such as captopril,[66] quinapril, or ramipril.[51] Indeed, tripeptides based on FQP should be selective inhibitors of the N- rather than the C-terminal active site. Presumably, the side-chain of glutamine (Q), $–CH_2–CH_2–CONH_2$, interacts with a deep cleft at the N-terminal site, unlike alanine's much shorter side-chain ($–CH_3$). This would explain why a hydrophilic FAP-like inhibitor like enalaprilat cannot bind well to the N-terminal active site.

Hydrophobic binding of the FQP tripeptide to the N-terminal active site may make mechanosensing by the N-terminal domain possible (Fig. 4). The FQP tripeptide may represent the C-terminal end of an intramolecular flap that binds hydrophobically to the N-terminal active site. The proline (P) at the C-terminus of the autoinhibitory domain (and of ACE inhibitors) may be required in order to disrupt any α-helix and allow the autoinhibitory domain to exit from the active site before bumping into the "stop" near the active site (Fig. 5).[5] The FQP-containing flap may be dislodged only mechanically by a shearing force (Fig. 4), rather than chemically by chloride, as in the C-terminal active site (Fig. 3).

There are a number of additional conserved differences between the N-terminal and C-terminal domains of the duplicated form of ACE. These may also contribute to keeping the N-terminal active site normally shut, since the N-terminal active site accounts for only about 25% of total enzymatic activity *in vitro*.[61] How additional conserved oligopeptides affect access to the N-terminal active site will have to await the crystal structure of the holoenzyme.

None of the ACE gene duplications before Chordata resulted in HExxH–FQP–HexxH–FKP (Table 3). For example, *Ance-2*::*Ance-3* in *Drosophila* would result in neutral tripeptides in both domains (FQP–FAP), making chloride activation impossible. In *Anopheles*, both domains had charged tripeptides (FEP–FKP in EAA09164), or the N-terminal active site was charged but not the C-terminal active site (VTD–FQP in EAA14498). Neither combination was the same as the evolutionarily successful vertebrate combination (FQP–FKP), perhaps explaining why none of the earlier gene duplications led to a closed circulation.

What is the experimental evidence for this hypothesis? The most convincing is the prolonged off-times[66] for hydrophobic ACE inhibitors such as quinapril and ramipril (about 24 h) relative to that of more hydrophilic ACE inhibitors like enalapril (about 4 h), which binds exclusively to the C-terminal active site. This suggests that the N-terminal active site may trap hydrophobic ACE inhibitors that bind in place of the FQP autoinhibitor tripeptide. The N-terminal site is likely to be a groove occluded by a "flap" containing the FQP tripeptide (Fig. 3).

In contrast, the "flap" occluding the C-terminal active site (here postulated to contain the FKP tripeptide) opens readily in response to chloride, resulting in a shorter off-time for hydrophilic ACE inhibitors that bind preferentially to the C-terminal active site (Fig. 3).

Before ACE was sequenced,[67] it seemed that there was only one active site that could bind a hydrophilic ACE inhibitor like enalapril.[68] This supports the concept that the N-terminal active site is normally occluded and relatively inaccessible to solvent *in vitro*.

Since the C-terminal active site is almost fully activated in the presence of 50 m*M* chloride, then it is constitutively active in the human circulation, where the plasma chloride concentration is 110 m*M*.[64] Only the N-terminal domain can be further activated *in vivo*. Activation of the N-terminal active site may be how ACE enzyme activity is regulated *in vivo*.

This would explain why hydrophobic ACE inhibitors that bind to both N- and C-terminal active sites are more effective than hydrophilic ACE inhibitors that bind to the C-terminal active site.[29] Perhaps systemic blood pressure depends on constitutive C-terminal peptidase activity (vascular tone based on enzymatic "tone"), whereas local signaling may be due to specific activation of the N-terminal active site

by turbulent flow. This could explain the occasional dissociation between target organ damage and the level of systemic blood pressure, as in "insidious" chronic renal failure with normal blood pressure.[69] And it would argue that N-terminal ACE inhibitors may be especially effective at preventing target organ damage from hypertension and diabetes.

This model could also explain the effect of cholesterol and saturated fatty acids on progression of atherosclerosis. Like the endothelial cell as a whole,[19–21] molecules on the endothelial plasma membrane are expected to orient in the direction of flow so as to minimize the cross-sectional area they present in the direction of flow. In laminar flow, the direction of flow is constant, and the cell (and presumably the molecules on its luminal surface) orients itself in the direction of flow. But in turbulent flow, the direction of blood velocity changes quickly. The cell remains polygonal, without elongating.[19–21]

With its axis of symmetry, ACE is likely to be an oblate spheroid (i.e., shaped like a lollipop), rather than a perfect sphere. In areas of turbulence, ACE will therefore orient so as to keep its molecular profile "stream-lined." But the molecule will not be able to rotate fast enough to keep up with the changes in the direction of the blood velocity if the velocity changes are rapid enough. The less fluid the membrane bilayer, the slower the ability of the ACE molecules to rotate in it.

Both cholesterol and saturated fatty acids lower membrane fluidity; they make the membrane bilayer less like olive oil and more like peanut butter. The rotational diffusion of a type I membrane protein like ACE (i.e., its ability to spin on its transmembrane stalk) will be decreased in a less fluid bilayer (Fig. 3). The result will be that more ACE molecules may be caught "broadside" by turbulent flow, unable to align themselves quickly enough in the direction of flow to avoid having their N-terminal active sites opened by shearing forces (Fig. 4). The result will be more ACE activation at any given blood velocity (and blood pressure).

The net effect of increasing the molar ratio of cholesterol and/or saturated fatty acids in the membrane will be that more ACE molecules will be activated for a given shear stress (blood velocity, or driving pressure). This effect will be magnified if ACE binds to any other molecule, which would act as a flywheel to increase ACE's rotational inertia and further slow its rate of rotation in the plane of the membrane bilayer.

Evolutionarily speaking, the rotational rate of the molecule had to be "tuned" to the forces encountered in the circulation. "Somatic" ACE, essentially a homodimer, has the requisite molecular mass. It is possible that the secretase that clips ACE from the membrane bilayer in endothelial cells (but not kidney proximal tubule) may bind to it, further increasing its effective mass.[70,71]

Extrapolating from ACE, other homodimeric proteases expressed on the endothelial plasma membrane, such as endothelin-converting enzyme,[6] may also function as mechanosensors in regions of turbulent flow.

NOTE ADDED IN PROOF

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